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# GASTRIC GLANDULAR TUMOURS PROVIDED WITH EXCRETORY DUCTS, AND CRITICISM OF THE THEORY OF THE TUMOURS ARISING IN HETEROTOPIC PANCREAS

*Observations on the occurrence of atypical glands in the stomach*

By

OSMO JÄRVI and PEKKA LAURÉN<sup>1</sup>

Received 30 vi 63

Some workers contend that the structure of gastric tumours merits no major attention as the structure may vary so much in the different parts of the tumour that one type of histological structure may be transformed into another (Stout 1943, 1953, Ackerman & del Regato 1962). The present authors however, hold that the structure also of gastric tumours warrants at least some conclusions concerning the origin of the tumours, and that the establishment of the type of tumour structure on which a classification may be based is of both theoretical and practical importance. Similar views were advanced *eg* by Borrmann (1926) and Willis (1960). In their study from 1951 on the histological structure of gastric cancer, the authors found that the tumours derived from metaplasias of the intestinal epithelium of the stomach in at least 20 per cent of the cases. This observation was later confirmed by several other workers (Mulligan & Rember 1954, Morson 1955, Wallenberg 1959 and Planteydt & Wulfighagen 1960). When the authors continued their studies in order to classify histogenetically all epithelial tumours of the stomach two tumours the histological structure of which differed distinctly from other tumours were separated from the material comprising more than one thousand investigated cases and classified into a special group. In both cases it was possible to distinguish in the tumour tissue a system of ducts opening into the mucosa as well as specific secretory cells forming glandular acini.<sup>2</sup>

## CASE REPORTS

*Case I* (601559) was a 44 year old wife of a worker with several years history of gastric trouble. She felt acute pain in the right epigastrium and in the side after

<sup>1</sup> The work of P. Laurén was supported by the Damon Runyon Memorial Fund. The authors thank Professor E. Vuori and Dr R. Järvi for providing the clinical reports on patients treated at Loimaa District Hospital and the Pori Municipal Hospital.

rich meals and her complaint had been treated as an acid dyspepsia. Gallstones were found at examination on March 2 1959 at the Pori Municipal Hospital and an operation was performed at which a single large stone was found in the gallbladder. Four months after the operation the patient began to complain again of pain similar to her preoperative pain. She had no appetite and lost 5 kg in weight in the course of the spring. At examination a mass the size of a small egg was palpable through the abdominal wall and a large round filling defect was revealed by X ray of the stomach. The gastric juice was deficient in acid total acidity being 7. At operation on August 7 1959 (Dr Jäfs) an egg sized round mobile tumour covered by an intact smooth mucosa was found in the stomach at the level of the angulus. The mucosa however was slightly irregular in some places. The tumour was removed. The patient made a good postoperative recovery but gastric pain recurred one month later. The patient was referred to the Surgical Clinic University of Turku where gastric resection was undertaken on October 26 1959. No new tumour tissue was found at the operation nor was such tissue demonstrable by microscopic examination of the samples. The patient last attended for a follow up on February 14 1961. She complained of back pain and micturition difficulties but no longer of abdominal pain. Nothing of note was found at the physical examination. Sugar metabolism tests were not carried out prior to the removal of the tumour. Nothing in the anamnesis suggested hypoglycaemia. No sugar was found in the urine. In a letter of September 4 1963 the patient reported that she was in good health.

Case 2 (3920/61) was a 33 year old fitter who after a few weeks history of pain of gastric ulcer type on April 30 1961 had an attack of severe upper abdominal pain lasting for about 1 hour. The patient was admitted to the Loimaa District Hospital where an operation was performed (Dr Hatanen). A perforated ulcer admitting a finger was observed close to the cardia in the lesser curvature and was closed with sutures. Microscopic examination of the excised specimen displayed carcinomatous tissue. Hence operation was repeated on May 15 at which tumour tissue was found below the cardia. In addition numerous enlarged lymph nodes were observed in the region between the stomach and the spleen. The case was considered inoperable and the operation wound was closed. At follow up examinations the patient was still in poor condition and unfit for work. He had lost some weight recently. X ray examination on October 5 1961 disclosed a distinct but fairly small sized ulcer niche in the body of the stomach. This was interpreted as a well demarcated cancer. The rest of the stomach looked normal. The patient was given several post operative courses of radiotherapy and from March 1962 was placed on 100 mg of Sendoxan per day. He was admitted to the Loimaa District Hospital with pneumonia on January 29 1962 and again to the Surgical Clinic University of Turku in October 1962. The case history included no symptoms associated with disorders of sugar metabolism. The blood glucose level on October 30–November 7 1962 was 85–110 mg per cent. The Wohlgemuth test gave 32 Wohlgemuth Units 45°/15 for urine and 4 W L 45°/15 for blood. Titz lipase was 0.40 T units. The glucose tolerance test was normal. Creatinine in the blood was 0.6 mg per cent. Alkaline phosphatase in the serum was 6.8 B L units. acid phosphatase 0.46 mg per cent. 17 ketosteroid determined in urine 15.5 mg/day. X ray examination on October 30 1962 revealed a crater of medium size in the posterior wall in the superior part of the body of the stomach. There was no characteristic walling off around the crater and it was not considered typical of malignant growth. X ray of the thorax revealed a considerable quantity of fluid in the left pleural sinus. The interlobar pleural boundaries were clearly visualized and paracardially to the left in the region of the inferior lobe dense infiltration which was interpreted as pneumonia. It was seen to extend laterally down from the hilus. At examination on November 28 the X ray finding in the stomach was unchanged. The patient however was in poor condition and died on December 8 1962. Unfortunately no autopsy was performed.

### *Histological Studies*

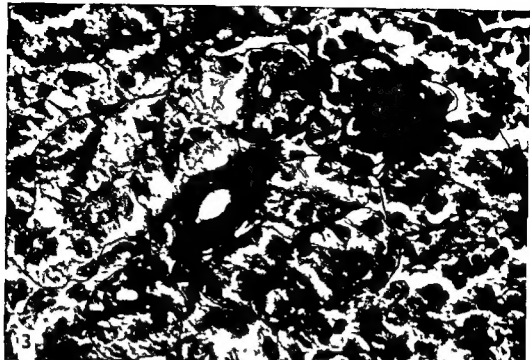
In case 1 the microscopic examination was performed on a specimen taken at the operation on August 7 1959 and in case 2 on a specimen taken at the operation on April 30 1961. The tumours differed so little from one another that a general description of their structure will suffice.



*Figs 1 and 2*

*Fig 1* (case 1) azan staining General view of the tumour A richly ramified duct opens into the mucosa at the right edge of the picture A part of the terminal acini are located in the basal portion of the mucosa There are large lobes of acinar tumour tissue in the submucosa  $\times 25$

*Fig 2* (case 1) azan staining red filter The epithelium of the ducts has intestinal columnar epithelium with brush border (e.g. high epithelium on the right) side by side with neutral mucous secreting gastric surface epithelium (cells staining dark grey apically) Goblet cells (dark isolated cells) are more numerous in the area of gastric than of intestinal type buds adjoin the ducts but usually also solid darker grey in section



*Figs 3 and 4*

**Fig 3** Case 1 PAS technique green filter Demilune shaped mucoserous terminal acini) or more complex adenomere structures adjoin the mucous tubules composed of neutral mucus containing cells The course of the basement membrane is accentuated with Indian ink in the middle of the picture the approximate contours of the intercellular secretory capillaries are accentuated similarly  $\times 350$

**Fig 4** Case 1, PAS technique The epithelium of gastric type in the ducts stains magenta with PAS Numerous fine secretion granules which have stained more faintly are to be seen in the cells of the terminal acinar conglomerates or long straight cell cords both often provided with secretory capillaries  $\times 200$



*Figs 5 and 6*

*Fig 5* Case 1 Masson Haemperl's technique Some enterochromaffin cells stainable with Masson's technique are encountered in the terminal acinar part of the tumour  $\times 350$

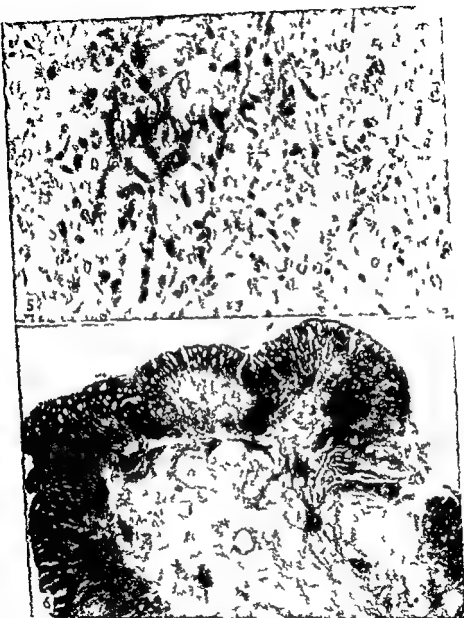
*Fig 6* Case 2 haematoxylin van Gieson staining A general view of the tumour To the right two of the ducts opens into the mucosa The mucosa of normal intestinal structure In contrast the submucosa shows numerous complexes of acinar tissue of the tumour adjoining the ducts  $\times 25$



*Figs. 3 and 4.*

*Fig. 3.* Case 1, PAS technique, green filter. Demilune-shaped mucoserous terminal acini or more complex adenomere structures adjoin the mucous tubules composed of neutral mucus-containing cells. The course of the basement membrane is accentuated with Indian ink in the middle of the picture; the approximate contours of the intercellular secretory capillaries are accentuated similarly.  $\times 350$ .

*Fig. 4.* Case 1, PAS technique. The epithelium of gastric type in the ducts stains magenta with PAS. Numerous fine secretion granules which have stained more faintly are to be seen in the cells of the terminal acinar conglomerates or long straight cell cords, both often provided with secretory capillaries.  $\times 200$ .

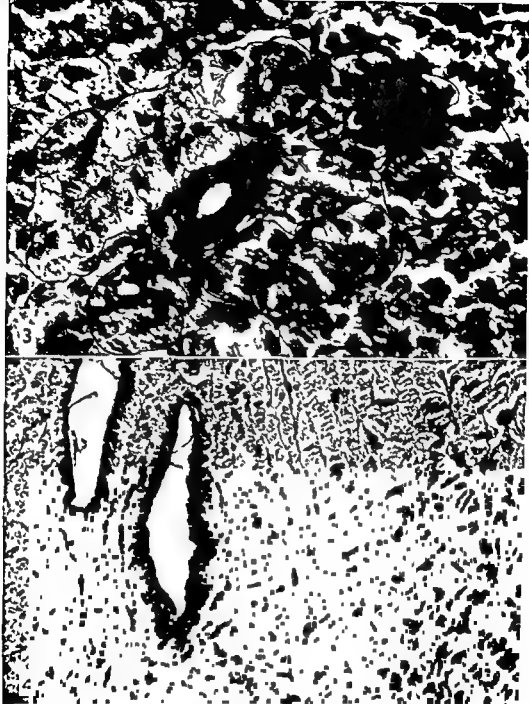


Figs 5 and 6

**Fig 5** Case 1 Masson Hammer's technique. Some enterochromaffin cells stainable with Masson's technique are encountered in the terminal part of the tumour  $\times 350$

**Fig 6** Case 2 haematoxylin-van Gieson staining. A general view of the tumour. To the right, two of the ducts open into the mucosa. The mucosa is of normal histological structure. In contrast the submucosa shows numerous complexes of fibrous tissue of the tumour adjoining the ducts  $\times 25$





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furthermore tubulus like terminal formations or narrow solid cell cords and rows which have no counterpart in the normal gland structure (Figs 2 and 4) The last mentioned may constitute extensive uniform tumour lobes with only thin septa of connective tissue between the cords (Figs 1-4 and 5)

It was possible to distinguish in the ducts primarily two types of epithelium one corresponding to gastric surface epithelium and one corresponding to intestinal epithelium The cells of the former type contained neutral mucus and stained magenta red with the periodic acid Schiff technique (PAS) they stained fairly intensely with Best's carmine but remained practically unstained with mucicarmine and alcian blue The intestinal epithelium again consisted of columnar cells with a brush border and of goblet cells irregularly spaced between the former cells The goblet cells contained acid mucus and stained bright red with the PAS technique medium blue with Heidenhain's azan trichrome they stained well with mucicarmine and alcian blue but were generally negative to Best's carmine Goblet cells could also occur in the gastric surface type epithelium of the ducts (Fig 2) The secretion of mucinous glands and tubules stained like the neutral mucus in antral and duodenal glandular cells It was negative with mucicarmine and alcian blue but stained rather heavily with Best's carmine It differed from the mucus of gastric surface epithelium by the bright red colour with PAS faint blue tint with azan stain The cells in the ducts however were not completely regular The nuclei could be irregularly grouped and variations in nuclear size were also observed Mitoses were also encountered occasionally

The cells of acini were generally conical with the apex directed towards the centre of the acinus Secretory capillaries separating the cells from one another were often found (Fig 3) The nucleus was round vesicular and located at the base of the cell The cells often contained secretion (Figs 2-4 and 7) In case 2 the secretory granules were larger and stained more heavily but there was no difference in principle between the cases The secretory granules stained moderately or faintly with PAS dark blue with azan weakly with mucicarmine and alcian blue but failed to stain with Best's carmine Case 1 showed acini with no demonstrable secretion in the acini In this case a few enterochromaffine cells were also demonstrated in some places in connection with the acini using the Hamperl-Masson technique (Fig 5) In addition using the Bodian technique in limited areas of acinar tissue a more diffuse brown black silver impregnation in small rod form was noted which was assumed to correspond to mitochondria Only scanty ergastoplasm was demonstrable in the cells whereas lamellae were encountered sometimes even supra-nuclearly In addition to secretory granules glycogen was noted frequently in the acini

The cellular structure was indicative of malignancy in both cases In addition to architectural disorders of form and composition of the ducts



Fig 7

Case 2 azan staining, red filter. Side branches adjoin the main ducts of the tumour and are connected with extensive terminal acinar complexes. Numerous blue staining secretion granules are to be seen in the cells of acinar tissue and partly in the duct cells as well  $\times 300$

In both cases the greater part of the tumour seems to have been located in the submucosa (Figs 1 and 6). In case 1 the mucosa was fairly well preserved and contained glands which were partly of body type and partly of antral structure. In case 2, in which the tumour was situated close to the cardia, the mucosa was of purely body structure. An area in which several ducts extending to the submucosa opened into the surface of the mucosa, however, were disclosed in both cases (Figs 1 and 6). The ducts ramified in the submucosa and partly already in the mucosal area, sometimes displaying dilatations of varying size. Smaller ducts departed irregularly from the main ducts and dilatations and ramified in turn (Figs 2 and 7).

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Anatomy, University of Turku, and comparison of the slides showed a similarity between the ductal structures seen in the cases reviewed by the present authors and those of the accessory pancreas. In addition, epithelium containing goblet cells and epithelium in which the cells contain neutral mucus resembling gastric surface epithelium, and, furthermore, pseudopyloric glands connected with the duct have been described together with epithelium secreting acid mucus in the ducts of normal pancreas (Zimmermann 1927, Gruber 1929, Feyrter 1931, Weinn & Kugler 1961). The acinar cells encountered in the tumours reported here, however, are poorly compatible with a pancreatic origin. The secretion of the cells differs clearly from the secretion of pancreatic exocrine cells and is mucoserous in quality. Ergastoplasm of the type encountered in pancreas cells was not observed and the centro-acinar cells of the neck parts which are characteristic of the pancreas were absent. On the other hand, the attention was attracted by the structural similarity of the cell cords encountered, especially in case 1 to those seen in tumours described as insuloma of the pancreas. However, mucinous or mucoserous secretion has not been reported in connection with the tumours.

Corresponding secretion granules in the tumour cells nor basal and supranuclear ergastoplasm nor intercellular secretory capillaries. Hence the similarity of gastric duct tumours reported in this paper and malignant insuloma was confined to the rough architecture, the tumours being completely different in their internal structure.

### *Relation of the Tumours to Glandular Abnormalities of the Stomach*

The following case may serve to throw additional light on the origin of the tumours.

Case 3 (made available to the authors by Dr H. Arko, Central Hospital of Kuopio) was a newborn male baby. A pea sized pale flat intumescence was observed in the ventral part of the stomach at the site of the greater curvature. Macroscopically (Fig. 8) showed the intumescence as a small, pale, flat, pea sized mass. The ducts were not enlarged.

A mass with azan partly blue partly red. The granules however were fairly large and resembled zymogen granules.

In this case the groups of cells adjoining the ducts resembled islets of Langerhans (Fig. 9) particularly the cell groups occurring in connection with pancreatic ducts, earlier reported by Feyrter (1939) and Baumann (1939) as 'insular duct organ'. A corresponding tumorous



and acinar tissue and occasional lack of secretory activity in the cells, nuclear irregularities were seen. The vesicular nuclei had a rough though loose chromatin network and the nucleoli were distinctly enlarged. Multinuclear giant cells with up to 24 nuclei were encountered occasionally. Here and there some mitoses were seen. In case 2 the tumour tissue grew distinctly inside the lymph vessels as well and penetrated the muscularis mucosae in many places from the submucosa, invading the basal parts of the otherwise well-preserved mucosa. In case 1 the tumour appeared to be better demarcated and it was not possible to demonstrate any growth in the lymph vessels. The cellular architecture, however, was more disordered in the acinar tissue in case 1 than in case 2.

To summarize the microscopic findings, both cases involved a highly differentiated tumour with exocrine secretion located principally in the submucosal layer of the stomach and draining into the mucosa through several ducts. No proliferation of the smooth muscle layer was seen to be associated with the tumour. The ductal parts of the tumour contained both gastric surface epithelium and intestinal epithelium and to a small extent also mucinous tubules of antral type. Terminal secretory acini which may constitute extensive irregular complexes or cords were associated with the ducts without intercalation of the neck parts. The acinar cells belonged to the group of mucoserous salivary gland cells with regard to their structure and secretion. The tumours were malignant in their histological structure, but obviously could have grown slowly and remained fairly well demarcated (case 1).

## DISCUSSION

### *Differential Diagnosis from the Tumours of Heterotopic Pancreas*

Comparison of the present cases with earlier literature may primarily suggest that they belong to the group of heterotopic (ectopic) tumours.

The best-known heterotopias in the stomach are the presence of intestinal epithelium in the gastric mucosa, the invasion of the submucosa by antral type (pseudopyloric) glands, and the occurrence of pancreatic tissue in different parts of the stomach wall. Cases in which pancreatic duct tissue is encountered without acinar tissue have also been described as adenomyomas or rudimentary accessory pancreases (Feyrter 1931). The occurrence of islets of Langerhans is also variable; they are frequently noted in exocrine gland tissue, occasionally they are absent, but cases have also been reported presenting ducts and islets only and no exocrine cells. No pancreatic tissue of normal structure was encountered in the present cases and all "heterotopic" tissue was tumorous. However, the ducts of the tumour were fairly similar to ducts occurring in accessory pancreases. Corresponding stainings were performed in the case of pancreas accessorium of the stomach, published in 1959 by Rintala from the Department of Pathological

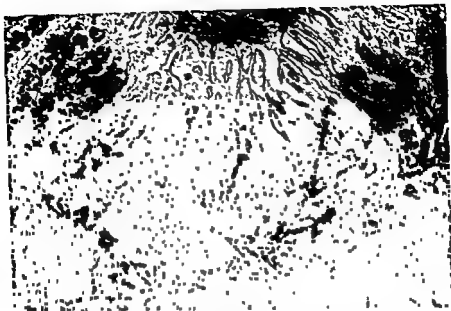


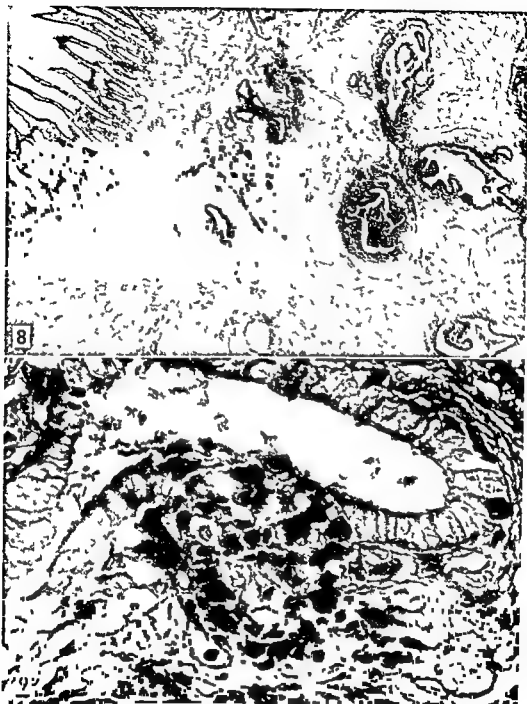
Fig 10

Case 4 PAS technique A gland group of peculiar type with serous and mucoserous cells, between normal antral glands in an ulcer stomach. The secretion granules stain moderately with PAS. To the right at the edge of the picture, also intestinal metaplasia in the epithelium  $\times 40$

growth in the pancreas was described as "nesidioblastoma" by *Laudlatov* (1938) and *Frantz* (1959). On the other hand, the cell groups might also have been as yet completely undifferentiated exocrine pancreatic tissue or still undeveloped initial tissue comparable with the acinar tissue of the tumours described in the foregoing. No doubt the tumours considered here might be assumed to derive from congenital development disturbances of this type.

It has proved possible in experiments to make the gastric surface epithelium penetrate the muscularis mucosae into the submucosa, e.g. by placing carcinogens in the stomach wall (*Howes & de Oliveira* 1948, *Stewart* 1953). *Järvi & Keyrilainen* (1955) and *Järvi* (1962) reported that neck epithelium as well as antral (pseudopyloric) glands were connected with the cavities thus formed. On the other hand, cells with mucoserous secretion have been encountered in the antral and duodenal glands of many different animals (*Schwalbe* 1872, *Bensley* 1903, *Villemun* 1920, *Tschassownikow* 1926, *Tehwer* 1929, *Burkl* 1950). This finding was confirmed by *Järvi* in mice<sup>1</sup>. Also in man glands may penetrate the submucosa or the deeper layers secondarily as so-called pseudopyloric heterotopic glands and, in addition, surface and neck epithelium in cases of giant hypertrophic gastritis (cf. *Järvi* 1962).

<sup>1</sup> Not published



*Figs 8 and 9*

*Fig 8* Case 3 haematexylin mucicarmine staining The mucosa to the left is of antral structure The muscle layer displays ducts of varying morphology consisting partly of pale higher gastric surface epithelium partly of darker intestinal epithelium There are in addition minor ducts in which the cells contain acid mucus and adjacent to them somewhat vaguely demarcated cell groups  $\times 40$

*Fig 9* Case 3 azan staining green filter A cell group reminiscent of islets of Langerhans adjoins the duct The cells contain granules staining partly blue partly red with azan  $\times 350$



*Figs 11 and 12*

- Fig 11* Case 2, hematoxylin van Gieson stain. Mixed seromucinous glands in the cardiac mucosa. The cells of the mucinous tubules contain neutral staining mucus, the demilunes and the acini are "mucoserous". The surface epithelium is partly intestinal type  $\times 30$ .
- Fig 12* A detail from Fig 11 showing the mucoserous demilunes and acini adjoining the mucinous tubules. The contours of the secretory capillaries in the former are accentuated with ink  $\times 300$ .

Furthermore, cells which differ from those described in textbooks and handbooks may be demonstrated in human gastric mucosa. Serous or mucoserous cells of this type have in general been assumed probably to represent only a certain secretory phase of antral glandular cells and they have, to a certain degree, escaped attention. The only exceptions are reported by *Hintzsche* (1939) and *Burkl* (1950), who described heterotopic serous acini and semilunes in connection with duodenal and antral glands in the stomach. In spite of the differing architecture of the cell grouping they suggested that acini might represent pancreatic tissue. *King & MacCallum* (1934) found one pancreatic acinus adjoining duodenal glands in a case with accessory pancreas in the pylorus. Here two examples are described of gland groups of divergent glandular structure located in the gastric mucosa.

*Case 4* (made available to the authors by Dr *Virtikunen*, Central Hospital of Northern Karelia) was a 56 year-old railway ganger. The patient had suffered from gastric pain for 4 years. X-ray revealed a gastric ulcer. A large gastric ulcer close to the cardia was found at operation. A specimen taken from the edge of the ulcer showed in the cardiac mucosa a gland conglomerate which differed from the environment (Fig 10). The glands were acinotubular. Secretory capillaries were seen between the cells. In their apical part the cells contained well preserved secretory granules which distinguished them from the antral gland cells, the secretion of which was reticular. The secretion in the cells was often divided into two groups: the supranuclear Golgi apparatus group and the group located at the cell apex. The granules stained, sometimes red with azan, sometimes purple or blue. With the PAS technique, however, they all gave a pale red reaction. Mucicarmin and alcian blue also produced a faint staining. The cells are consequently comparable with the serous and mucoserous cells of the salivary glands. No differentiated ducts were associated with the glands which drained directly into the foveolae of the mucosa.

*Case 5* (made available to the authors by Dr *Slatis*, Central Hospital of Vaasa) was a 41 year old policeman presenting a years history of gradually increasing pain in the abdominal region and occasional vomiting. X-ray examination disclosed an extensive constriction in the body of the stomach, a mass was palpated also in the epigastrium. At operation a constricting tumour was found in the body of the stomach. Microscopic examination revealed a scirrhous carcinoma. The gastric surface epithelium was displaced by intestinal epithelium over wide areas.

The second specimen, in which no tumour tissue was disclosed, taken from a site close to the cardia, revealed an atypical gland conglomerate about 15 mm in diameter in the basal part of the mucosa. The glandular tubules had lost their usual vertical course in the mucosa and ramified freely in the environment (Figs 11 and 12). Demilune-shaped terminal cell groups or fully developed acini were found in many places in association with the mucinous tubules. The mucinous cells of the tubules corresponded in structure and in the stainability of their secretion to cells of the cardiac (as well as antral) mucosal glands. The mucus stained well with Best's carmine and bright red with PAS, it did not take mucicarmin or alcian blue and stained faintly blue by the azan technique. The acinar cells, however, had a conical shape, the apex pointing to the narrow central lumen, the cells were separated from each other by secretory capillaries. The nuclei were round and not flat as is often seen in mucinous cells, a weakly stainable ergastoplasmic lamellar system was found both basally and in the supranuclear zone. The cell cytoplasm contained well preserved secretory granules. These stained with various brown colours in haematoxylin van Gieson stain, negative with Best's carmine and moderately positive by the PAS technique.

In these cases, accordingly, foci of acinotubular glands clearly deviating from normal antral, cardiac or body glands occurred in the cardiac mucosa. Because of the neutral character of the mucus of the



mucinous cells, the glands in the latter case resembled primarily the duodenal seromucinous glands occurring in some mammals. No special duct system was present in these cases.

The atypical glands found in the two cases described here closely resemble the glands occurring in the cases reported by *Hinzsche* and *Burkl*. The writers, however, are not inclined to follow these authors in interpreting the serous cells as pancreatic. *Burkl* points out some divergency in structure of part of the cells and the strange way in which the serous cell groups adjoin the mucinous tubules, which is comparable rather with a seromucous salivary gland than with pancreas. It may be mentioned that *Jarvi & Meurman* (1964) had observed pancreatic acini associated with metaplasia of gastric surface epithelium in the gall bladder mucosa, but the cell structure and grouping of the cells into acini seen in this case differed markedly from the cases described above. It is interesting to note, however, that *Burkl* was able to collect from among 47 pathological gastric specimens a total of 8 cases presenting these atypical glands, he suggested that the glands had arisen as acquired metaplasia resulting from moderate gastritis. Further, he found some intercalated ducts adjoining these glands, which rather strengthens the comparison with the tumours provided with a ductular system reported in this paper, though it can hardly be denied that there is a difference as regards the extent to which the ducts in both categories have developed.

Thus, considering the above cases it seems possible that mucoserous acinar tumours with ducts in the gastric wall may arise in the following ways: (1) on the basis of a congenital developmental disorder involving some salivary type gland or possibly pancreatic tissue; (2) on account of some irritation of the mucosa and wall of the stomach from which glandular metaplasia might also ensue. In this phase of the investigation, it remains an open question whether one or the other of these alternatives apply.

### *Review of the Literature on the Solitary Gastric Tumours Suggested to Have Arisen in Heterotopic Pancreas*

The connection in one way or another of the tumours described above with accessory pancreas is supported by reports in the literature which however do not take other alternatives into consideration. In 11 cases the tumour is assumed to originate from pancreatic tissue in the stomach but the reasons for such an assumption are vague in all of the cases.

In the case reported by *Pfforinger* in 1904 the solid carcinomatous tissue seemed to be of submucosal origin. There was a great deal of accessory pancreatic tissue in the duodenum but the tumour was not found to be connected with it at any site.

In 1908 *Branham* reported a 20 year old patient with a tumour in the pylorus. The tumour was first diagnosed as a polycystic carcinoma but *Prof. W. H. Welch* interpreted it as a malignant adenoma derived from an accessory pancreas. As *Welch's* statement allows of several interpretations the essential passage is cited here. In the submucous coat is a granular mass presenting the structural features of the pancreas with certain deviations from the normal type. There are spherical and oval acini lined or filled with cubical or cylindrical granular epithelial cells; these acini corresponding in shape and general appearance to those of the pancreas and unlike

any cancerous or adenomatous growth originating from the gastric tubules. These tubules are not in connection with this submucous growth. The granular mass consisting originally of the misplaced pancreatic tissue is the starting point of a true tumor formation characterized by irregular alveoli usually lined but sometimes

no question that it is composed entirely of carcinomatous pancreatic epithelium" ing possibility is

of cystal origin. The symptoms also and the cystic duct were absent. On the basis of these arguments the author assumed that the tumour started from an accessory pancreas but he was unable to detect it.

Further *Borrmann* (1926) mentioned briefly the finding of a solid carcinoma which might have derived from pancreatic tissue but failed to display any definite signs indicating an

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In a table of their material of accessory pancreases *Barbosa et al* (1946) listed a case in which the pancreas was located directly below a carcinomatous ulcer. Malignant change of accessory pancreas was suggested but no description of the tumour was given.

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In 1963 *Hornay & Buday* established the diagnosis of a heterotopic adenomyosis which had become malignant at the site of a gastroenteroanastomosis 40 years after gastric intervention.

According to reports of tumours in the stomach



## *Review of the Literature on the Solitary Intestinal Tumours Suggested to Have Arisen in Heterotopic Pancreas*

A number of tumours similar to those described in the preceding paragraph have been found in the lower parts of the gastrointestinal tract

A cystic degenerated carcinoma in the duodenum the structure of which resembled pancreatic tissue was reported by *Bookman* (1932), but the article gives little information about the tumour

In 1943 *Duff et al* described a duodenal adenocarcinoma which on the basis of the deep location in the duodenal wall and of the microscopical structure of the tumour they thought to be comparable with the pancreatic ducts regarded as deriving from accessory pancreas

Further *Holman et al* reported in 1943 an apparently hormonally quiescent case presenting a tumour structurally of the islet type and located in the duodenum No pancreatic acinar or ductal tissue were seen

In a case reported by *Brown et al* (1944) a stenosing tumour of the ascending portion of the duodenum was resected Microscopic studies of the mass showed a new growth arising from pancreatic tissue Since the pancreas was normal it was concluded that the tumour originated from accessory pancreatic tissue in the duodenal wall (which tissue apparently could not be demonstrated) The character of the tumour strongly suggested that the aberrant tissue was ductal in origin

One case is included in the table published by *Barbosa et al* in which an accessory pancreas is overlying a carcinomatous duodenal ulcer at the outlet of the common bile duct Malignant change of accessory pancreas was suspected

*Silver & Lubliner* (1948) in connection with their report of 104 pancreatic tumours briefly mention a 80 year old woman with the clinical diagnosis of gastric malignancy At autopsy an adenocarcinoma was found which was reported as having arisen in an accessory pancreas of the duodenum The authors do not consider the case as islet cell tumour

*Oberhelman et al* (1958) reported a patient who repeatedly had gastric ulcers A non insulin producing islet type tumour in the duodenum with suspicion of lymph node metastasis to the region of the head of the pancreas was found Apparently normal pancreatic tissue was absent in the duodenum but the figure of the lymph node shows in addition to islet type cords distinct acinar tissue though whether tumorous or not cannot be evaluated In the discussion the authors mentioned one more case of duodenal non insulin producing islet cell tumour associated with clinical steatorrhea syndrome

For completeness it may be added that the 7 cases by *Scagliosi* (1913) described as duodenal carcinomas or beginning carcinomas arising in connection with the accessory pancreas presented evidently no true tumour as *Feyrier* (1931) had already stated In all of these 7 cases the suggested tumour of microscopical size represented an additional finding at autopsy

In a very poorly described autopsy case *Seidelin* (1912) found a small carcinoma in the jejunum peripherally presenting a structure of pancreatic tissue

*Herrheimer* (1913) described an extensive carcinoma in the small intestine which in his opinion had arisen in an accessory pancreas

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## *Non-Insular Tumour Cases Suggested to have Arisen in Heterotopic Pancreas Outside the Stomach and Intestine*

The authors have found only three cases of this category described in the literature *Silver & Lubliner* reported an autopsied case A 53 year old man had an

— of the accessory pancreas in the porta hepatis with numerous histologic description was given Another case with  
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porta as well as the cystic and hepatic duct were

The study confirms earlier observations that these ruptures occur independent of age and pathologic changes of the aorta. Middle aged men form the largest group probably because they most often expose themselves to serious traffic accidents. In contrast to earlier observations the material contains more motorists than pedestrians. This is probably because most accidents happened on country roads where as civilization proceeds pedestrians are a vanishing race.

Traumatic rupture of the aorta is usually accompanied by fatal intrathoracic haemorrhage. The survival time is only in a few instances long enough to permit surgical treatment. In a number of cases this injury is the single serious lesion in cases of traffic accidents. There are therefore ample reasons to analyse as far as possible the mechanical forces acting in these instances in order to find adequate counter measures. Such analysis is the subject of another study (In detail 1964).

### SUMMARY

Twenty one cases of fatal traumatic ruptures of aorta are reported. All cases except one were traffic accidents. Middle aged men were the largest group of the injured. The ruptures were predominantly located at the isthmus of aorta. In 16 cases only one rupture occurred, in 5 cases there were 2 or more ruptures. Severe intrathoracic haemorrhage was present in all cases and probably represented the single cause of death in all cases.

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In the case reported by *Ross* (1933) a solid adenoma with cells resembling partly acinar partly islet cells of pancreas but presenting no ducts caused obstruction of the small bowel

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The authors have found only three cases of this category described in the literature. *Silver & Lubliner* reported an autopsied case. A 53 year old man had an

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Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents. Even Kleinsasser (1943), Strassmann (1947) and Tannenbaum & Ferguson (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture. Lellner (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart's downward traction on the aorta

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest, and Kleinsasser (1943) sustained this opinion

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture. Tannenbaum & Ferguson (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance those cases in which rupture is caused by flying pieces of wood or stone hitting the chest

Marshall (1938) pointed out that when the body is decelerated during forward motion the hilus of the left lung will exert a traction on the parietal pleura covering the aorta. By this mechanism aorta is probably kinked in such a way that rupture occurs

Cammack et al (1959) found that antero-posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region

To summarize, earlier authors agree that traumatic rupture occurs in connection with a considerable and sudden change of velocity of the body. The direction of the velocity change may vary from one case to another and is often but not always accompanied by a contusion of the chest. There is no agreement why the isthmus region is most exposed to ruptures

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-

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## THE MECHANISM OF TRAUMATIC RUPTURE OF THE AORTA

By

JON LUNDEVALL

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Traumatic rupture of the aorta may occur when the body, or the upper part of the body, is exposed to a sudden change of velocity by blunt force. The ruptures usually occur in the isthmus region, more rarely in the ascending part of aorta, and seldom in other parts of the vessel. The traumatic ruptures are nearly always transverse. (For references, see *Lundevall 1964*)

The detailed mechanism which produces these ruptures have been discussed for many years, and opinions have in part been contradictory. The aim of this study is to give a survey of the different views, and to contribute to the discussion by personal observations.

### *Earlier Investigations*

*Rindfleisch* (1893) held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching.

*Oppenheim* (1918) ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg. By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves. In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point. In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg. Dissecting aneurysms frequently occurred during the experiment. In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents. *Oppenheim* admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death. The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered.

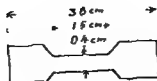


Fig 1  
Dumbbell specimen

### Observations on the Resistant Power of the Aortic Wall

Specimens of standardized measures (Dumbbell specimens, Fig 1) were punched from each of the 10 aortas already described

From each aorta, four specimens (Nos 1-4) were taken from the p asc, with their long axis along the blood stream, and symmetrically distributed around aorta's circumference. The next four specimens (Nos 5-8) were from the isthmus region, and were also in symmetrical positions around the circumference, their long axis parallel to the blood stream. The last two specimens (Nos 9-10) were from the posterior and the lateral parts of aorta's wall a little below isthmus, and were also taken from the lower thoracic portion of each of 2 aortas. In each case, one specimen was longitudinal, the other transversal to the axis of the vessel, at the same level.

Adventitia was removed from the specimens, and the thickness of the wall was measured with a micrometer in two specimens from each aorta, one from p asc, and one from the isthmus region.

The specimens were stretched until they ruptured in an Instron stretching machine, kindly placed at disposal by the Norwegian Institute for Industrial Research. The rate of stretching was the machine's fastest, 50 cm per minute. The load at the moment of rupture was recorded. The results are summarized in Table 2.

TABLE II  
*The Tensile Strength of the Wall of Aortas from 10 Bodies*  
*Load (grams) of Dumbbell Specimens at Moment of Rupture*

Body No	Spec no (p asc)				Spec no (isthmus)				Spec no (p desc)		Lower (p desc)	
	1	2	3	4	5	6	7	8	9	10	Long	Tr
I	530	490	730	400	490	520	360	190	400	320	410	895
II	375	735	825	455	505	215		315				
III	845	690	720	800			370	335	400	320		
IV	525	460	555	590	555	365	430	265	430	455		
V	560	440	540			485	235	200		240		
VI	690		545	390	355	250	365	310	315	365		
VII	670	440	490	560	535	430	390	320		650		
VIII		495	575	560		310	330	285				
IX	590	350	335	625		640	440	315	490	380	885	595
X	375	300	620	520	280	190	125	220	400	245		
Mean values		547.8				350.8				397.4		

ing velocity changes, and the ability of the aortic wall to resist these forces. The following observations were made in order to obtain some data for this discussion.

## MATERIAL

Aortas from 10 bodies were studied. The bodies were of 7 men and 3 women aged between 35 and 84 years. Nine of the patients died of disease, one (the youngest) of barbiturate poisoning. All aortas presented various degrees of atherosclerosis but none had calcifications of the aortic wall. The observations were made 1-2 days after death.

## Anatomical Observations RESULTS

The course of the aortic arch deviated 20-25° from the sagittal plane. In each case, the thoracic part of aorta was relatively mobile, but the isthmus region was slightly more fixated to its surroundings than the portions of aorta above and below. The great arterial branches from the arch did not particularly restrain aorta's movements, nor did the intercostal arteries. The arterial branches were easily stretched. The recurrent nerve, running close to the concavity of the aortic arch at isthmus, was fairly resistant to traction. In some instances the nerve was readily palpable through the aortic wall when aorta was pressed against the pulmonary root.

The heart was relatively resistant against vertical movements, but was somewhat more mobile transversally, and could also to some extent be rotated along its long axis.

The root of the left lung was relatively well fixed against any movements. The anatomical measures of the aortas are given in Table 1.

TABLE 1  
*Anatomical Measures of Aorta from 10 Bodies*

Body			Arch diam cm	Aorta's radius cm		Thickness of wall cm	
No	Age (years)	Sex		base	isthmus	base	isthmus
I	35	♂	8	0.9	0.7	0.125	0.12
II	36	♂	6	1.0	0.8	0.155	0.165
III	39	♀	8	0.7	0.6	0.18	0.155
IV	47	♂	11.5	1.2	0.9	0.25	0.18
V	64	♂	10	1.4	1.1	0.215	0.2
VI	64	♀	8	1.3	1.0	0.14	0.14
VII	68	♂	10	0.9	0.6	0.18	0.13
VIII	79	♂	11	1.2	1.0	0.225	0.185
IX	80	♀	9	1.0	0.8	0.20	
X	84	♂	7.5	1.4	1.2	0.18	0.115
Mean values			8.9	1.1	0.87	0.185	0.167

Arch diameter is the horizontal distance between the outer (medial) wall of base and the outer (lateral) wall in the isthmus region at the level of the top of the concave wall of the arch.

Aorta's radius at base and isthmus was calculated from the measured circumference.

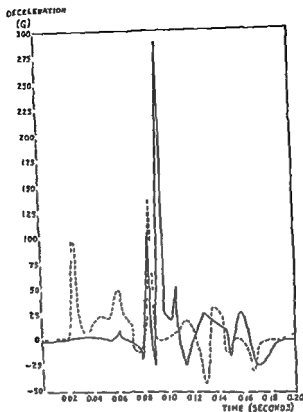


Fig 2

Deceleration/Time diagram (From Odelgard & Weman 1957)

--- Automobile      ——— Shoulders

There are three kinds of mechanical forces which may act on the aortic wall during rapid velocity changes

- A Traction and pressure on aorta by other thoracic organs, and torsion of the aorta
- B Strain waves in the aortic wall
- C Pressure variations in aorta's blood content

These forces will be discussed on the basis of the observations referred, and other author's observations

1 *The effect of traction and pressure on aorta by other thoracic organs and torsion of the aorta* From the anatomical observations it appears that during sudden changes of velocity in the sagittal plane (as in the example studied by Odelgard & Weman 1957) both the arch and the lower thoracic portion of the descending aorta are probably dislocated forwards or backwards in relation to the more fixed areas at isthmus and the base of the heart. At these two points therefore,



During the tests, a few specimens were crushed at the fixation points, and were omitted as indicated in the table

During the slow stretching, the specimens were considerably elongated and reduced in thickness before they ruptured, and did not regain their original thickness when the stretching was discontinued. The resistance of the specimens against the stretching was therefore mainly due to viscosity, not to the elasticity of the tissue.

The resistant property of the wall varied considerably around the circumference at each level within each aorta. However, the resistance was mainly less at isthmus than in the ascending and descending parts, and the transversal specimens resisted twice the load which caused rupture of the longitudinal specimens.

## DISCUSSION

### 1 Forces Acting on the Aortic Wall

A typical accident in which rupture may occur is the case of a motor car driver who is thrown against the steering wheel as the car collides frontally. This situation has been studied experimentally by Odelgard & Weman (1957). In their experiment a dummy of anthropometric measures was rigged in the driver's seat of a car with a safety harness. The car was dropped vertically and hit the ground at a speed of 60 km/h. The movements of the car and the dummy were recorded, and derivations of the recordings gave, among others, the diagram shown in Fig. 2.

The dummy's shoulders were subjected to heavy vibrations lasting for about 0.12 seconds, during which decelerations up to 290 G occurred (G expresses the degree of change in velocity in multiples of magnitude of the acceleration of gravity). Other recordings showed that the hips of the dummy were also vibrated, but not synchronous with the shoulders. The authors pointed out that, owing to the elastic response of the individual's body and the car's wheel, the magnitude of body vibrations on impact is difficult to predict. It is reasonable, however, to assume that the absence of a damping harness will accentuate the peaks of deceleration, and also that these peaks will rise with the collision speed.

The internal organs, including the aorta, will presumably also be exposed to vibrations during an impact. The magnitude of these vibrations, however, is unknown at present because no experimental studies have been reported. Some authors referred by Zehnder (1960), have made quantitative calculations of the degree of deceleration or acceleration necessary to produce rupture. These calculations are based on the assumption that the change of velocity of the body, and of the aorta, is steady during an impact, but Odelgard & Weman's experiments have shown that this probably is not the case.

authors already referred have stated that a sudden increase of the blood pressure is an important factor in the mechanism of traumatic rupture of the aorta no direct observations in this pressure have been reported. The discussion of this factor must therefore be based on theoretical considerations.

A local rise in the blood pressure may be caused in two ways. Firstly by a local compression of aorta followed by displacement of the blood with an expansion of the adjacent uncompressed parts of aorta. Secondly by the propagation of a pressure wave in the blood content causing a waterhammer effect.

The first of these mechanisms is contingent upon the compression being applied for a time long enough to allow the blood to flow away from the compressed area and the rate of application of the pressure being so fast that only a limited area of the aortic wall at both sides of the compression is expanded. Woritz (1932) found that pressures of 800-1000 mm Hg applied in the aorta of living rats caused rupture of aorta or its branches. Aorta did not rupture because the blood was led away too fast. It is difficult to imagine that this mechanism of blood pressure increase can produce a single circular rupture as is usually seen in accident cases.

The second mechanism is illustrated by the following example. If a person during forward movement is suddenly stopped the blood in the aortic arch will continue forwards by its inertia and exert a pressure against the anterior wall of the ascending part of aorta and a corresponding traction on the posterior wall in the isthmus region. The pressure is proportional to the weight of a horizontal column of blood lying close to the concave inner surface at the top of the arch and which has a base of 1 sq cm. From the mean of measures of 10 aortas referred in Table 1 and the blood's specific weight it was calculated that this column weighs 9.4 g. At a given degree of velocity change expressed in G's the pressure is therefore 9.4 G g/sq cm.

According to Wehn (1963) the resulting tension at different points of the aortic wall can be calculated in the following way.

The aortic arch can be regarded as the half of a ring formed tube as shown in Fig. 3.

The ring's axis is the line AB lying in the plane of the paper.

The distance in the point E is found by tracing a diameter through this point and prolong it to the axis AB. R is the distance CF and its plane of rotation is vertical to the paper's plane.

Corresponding to the line FG the radius of curvature (R) shifts from  $+\infty$  to  $-\infty$  which means that R referring to points on the concave side of the tube's surface has a negative sign. The point D therefore had a radius of curvature (R) equal to  $-CD$ .

The formula for circular (meridional) stress ( $L_1$ ) is (Wehn 1963)

local longitudinal stretchings of the aortic wall occur. In cases in which the antero-posterior diameter of the chest is reduced (as when the chest is pressed against the steering wheel), the heart will probably be dislocated to some extent to the left and also be rotated along its long axis. A tangential torsion stress will then act on the aorta, preferentially on the part just above the semilunar valves, and tend to produce a spiral-formed rupture.

During velocity changes along the vertical axis of the body (as when the individual falls on his feet or buttocks), the arch and the descending part of aorta will probably be dislocated downwards in relation to the isthmus region, producing a stretching of the upper lateral part of the wall at isthmus. The arch will then be pressed against the left hilus, and the recurrent nerve may execute a cutting pressure from below.

In traffic accidents, abdominal injuries often occur, causing an increased intra-abdominal pressure. The diaphragm is often ruptured, and abdominal viscera are occasionally displaced into the left thoracic cavity. The left lung's hilus is then pressed upwards, resulting in an increased bending, or even kinking of the aortic arch. This mechanism will therefore tend to produce a transverse rupture in the isthmus region.

The cervical vessels are stretched when the head is moved backwards, or when the aortic arch is pressed downwards. Even if these vessels are easily stretched, they may exert a local traction on the aortic arch.

In most accidents, the movements of the body are probably complex, the directions of velocity changes varying during fractions of a second. Particularly in cases in which a pedestrian is hit by a car, he often tumbles head over heels, and it is virtually impossible to reconstruct which of the stresses discussed is the predominating in the mechanism causing aortic rupture. It will also be apparent that the magnitude of the forces at present is incalculable.

*B Strain waves in the aortic wall* Aldman (1962) has shown that when an elastic belt is stretched, a strain wave, which is a local elongation of the material, will start at one anchoring point of the belt. The wave travels along the material and is reflected at the belt's ends. If the belt is stretched faster than the material can be elongated, the belt will rupture. The lowest velocity at which rupture occurs by this effect is called the critical velocity, which can be estimated when the velocity of the strain waves are known.

It may reasonably be assumed that similar strain waves occur in the elastic wall of aorta in accidents where a part of aorta is suddenly stretched. As no experiments have been made to estimate this condition, the importance of the phenomenon for the causation of rupture of the aorta is still unknown.

*C Pressure variations in aorta's blood content* Although several

The formula for longitudinal stress in a straight tube is (Windsor 1959)

$$L_2 = \frac{p r}{2} \text{ g/cm}$$

and the corresponding tension ( $S_2$ ) is

$$S_2 = \frac{p r}{2 h} \text{ g/100 cm}$$

Wehn (1963) has calculated that this tension has the same value in a curved tube (the hoop tension) as in a straight one, and the tension therefore, is independent of  $R$ .

The numerical values for  $S_2$  at different points were calculated from the data already referred, and were found to be

$S_2$ at isthmus	$355 + 25 \text{ G g/100 cm}$
$S_2$ at aortic	$400 + 28 \text{ G g/100 cm}$

In the outlined situation, the waterhammer effect will therefore cause a tension in the aortic wall which is greater in aortic than in the isthmus region, and the longitudinal tension (which would produce a transverse rupture) is about half as great as the circular tension.

## 2 The Resistant Power of the Aortic Wall

Zehnder (1960), who measured the traction tolerance of specimens from aortas from 27 bodies found that the tolerance varied considerably among individuals, and also within each single aorta. The tolerance was generally less against traction along the blood stream than against transverse (circular) traction. Although the rate of onset of traction was not stated, the author concluded that the percentage elongation of the specimens before they ruptured expressed the elasticity of the aortic wall.

In ideal experiments, the resistant power of the aortic wall should be tested during conditions similar to those occurring in actual accidents. The tests should therefore be done on living tissue, and stress should be applied with a rate of onset and a duration as would occur in an accident. This was not feasible during laboratory conditions, and for this reason a method similar to Zehnder's was employed.

In the experiments referred, only the resistance due to viscosity of the tissue was measured. During actual accidents, the stress on the aortic probably sets in so rapidly that there is no deformation of the wall before rupture occurs. A resistance due to viscosity is then excluded, and the experiments do only coarsely reflect the resistant power of the aortic wall during such accidents.

$$L_1 = p r \left( 1 - \frac{r}{2R} \right) \text{ g/cm}$$

where  $p$  is the pressure acting on the wall from the inside. On the convex side  $L_1$  is less than  $p r$ , while on the concave side  $L_1$  is greater than  $p r$ . The circular stress has a maximum on the concave side of the tube's wall and a minimum on the convex side.

The tension on the wall ( $S_1$ ) produced by the stress ( $L_1$ ) is defined as the stress divided by the wall's thickness ( $h$ )

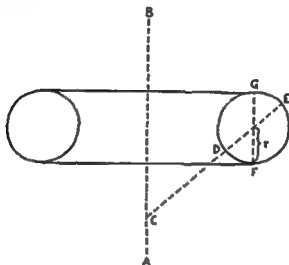


Fig 3

The aorta as a curved tube

For numerical evaluation of the circular tension caused by the waterhammer-effect in the mechanism outlined above, the mean values of  $R$ ,  $r$  and  $h$  in Table 1 were used, although  $R$  and  $r$  probably are greater *in vivo* than post mortem, because of a possible postmortal contraction of aorta. The pressure  $p$  is the sum of the physiological blood pressure (about 100 mm Hg or 136 g/sq cm) and the additional pressure caused by the waterhammer-effect, 9.4 G g/sq cm.

From the formula

$$S = \frac{p r}{h} \left( 1 - \frac{r}{2R} \right)$$

the following values were found

$S_{1 \text{ max}}$	at concave side of isthmus	811 + 56 G g/sq cm
$S_{1 \text{ min}}$	at convex side of isthmus	639 + 44 G g/sq cm
$S_{1 \text{ max}}$	at concave side of p asc	956 + 66 G g/sq cm
$S_{1 \text{ min}}$	at convex side of p asc	709 + 49 G g/sq cm

region. From the study of *Odelgard & Weman* (1957) we know that in an autocar collision the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above. It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture.

The waterhammer effect alone would theoretically cause circular and longitudinal ruptures at about equal degrees of velocity change. *Schnurbein* (1926) found that when increased internal pressure was applied in the straight portion of the descending aorta in rabbits the ruptures which occurred were longitudinal not transverse. Why then are the ruptures occurring in aorta in actual accidents nearly always transverse? The reason probably is that the other forces already discussed which tend to cause transverse ruptures act together with the waterhammer mechanism.

A study of the mechanism of traumatic rupture of the aorta is necessary for the choice of adequate countermeasures. There is probably not much we can do to protect the pedestrian when he is hit by a fast moving car but the motorist driving a car which collides can be protected to a certain degree against this serious injury. The aim of this protection must be to reduce the rate of deceleration and the development of adequate devices should be encouraged. The ideal design of such devices depends on a precise knowledge of the mechanism which causes the damage.

## SUMMARY

The mechanism causing traumatic rupture of the aorta is discussed on the basis of earlier observations and personal studies. The basis for the discussion is at present however insufficient and the conclusions must be drawn with caution.

Of the different forces acting on the aortic wall only the effect of a pressure wave in aorta's blood content (a waterhammer mechanism) could be approximately calculated.

Then tensile strength of the aortic wall of human bodies was tested under static conditions. The results indicated that the isthmus region is less resistant to stress than the ascending and descending parts of aorta.

On comparing the results it is concluded that rupture of the aorta is probably caused by the effect of forces of different kinds. A waterhammer effect probably plays an important role but local pressure and traction and strain waves in the wall act simultaneously and explain why the ruptures are nearly always transverse and preferentially located in the isthmus region.

Secondly, dead tissue probably has a higher power of resistance than living tissue (*Oppenheim* 1918 and personal observations) *Schnurbein* (1926) found that aortas of rabbits ruptured at about 1.3 atm internal pressure, whether the rabbits were freshly killed or already had a well developed rigor. All his animals, however, were dead at the time of the experiments.

These objections mainly concern the absolute values of resistance. The measures probably give an approximately reliable picture of the relative resistant property of the different areas of each single aorta. A calculation of the resistant power of the different parts of aorta, based on the mean values from Table 2, seems therefore justified.

By derivation of the results it was found that the main resistant power of a cross section of the aortic wall against longitudinal traction is

at isthmus	5250 g/sq cm
at p asc	7400 g/sq cm

It was therefore concluded that the tissue of the aortic wall is somewhat less resistant to slow traction in the isthmus region than in p asc. It was also presumed that the resistant power against transversal traction is the double of these figures in the respective areas, although this was measured in only two of the aortas.

*Comparison between damaging forces and the resistant power of the aortic wall.* From the anatomical observations it appears that traction and pressure on aorta by other thoracic organs, torsion of the aorta, and strain waves in the aortic wall during sudden stretching will mainly tend to produce transverse ruptures. The observations indicate that the ruptures caused by these forces preferentially would occur in the isthmus region, but also in some case in the ascending part of aorta.

In a certain situation, commonly occurring during accidents leading to rupture of a aorta, a waterhammer-effect caused by a pressure wave in the blood of the aortic arch must also be taken into consideration. The amount of this effect rises with the degree of velocity change, and by derivation of the mean of the experimental results it was found that the waterhammer effect alone would produce rupture of the aortic wall at the following points, and at the following degrees of velocity change.

Transverse rupture at isthmus	at 196 G
Longitudinal rupture at concave side of isthmus	173 G
Longitudinal rupture at convex side of isthmus	224 G
Transverse rupture in p asc	250 G
Longitudinal rupture at concave side of p asc	210 G
Longitudinal rupture at convex side of p asc	286 G

Although the figures are based on observations subject to criticism, it is reasonable to conclude that a somewhat higher degree of velocity change is necessary to produce rupture in p asc than in the isthmus.

region. From the study of Odelgard & Weman (1957) we know that in an autocar collision the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above. It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture.

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## A FACTOR IN THE LIVER SCATTERING AGGREGATES OF LYMPHOID CELLS IN ARTIFICIALLY PREPARED SUSPENSIONS

By

K. E. FICHTELIUS

Received 27 II 64

It was by chance observed that the number of cells in suspensions of thymus and lymph gland cells increased on the addition of liver extract. Regardless of the type of liver factor concerned here, this observation sheds some light on the possible errors that may arise if the number of cells is calculated in artificial suspensions.

### THE NUMBER OF SUSPENDED LYMPHOID CELLS BEFORE AND AFTER THE PASSAGE OF GLASS BEADS COATED WITH ORGAN EXTRACTS

#### *Experiment 1 A*

In experiments involving the transfusion of  $P^{32}$ -labelled thymus cells and lymph gland cells, Fichtelius (1958 a and b) demonstrated differences between the two types of lymphocytes. Labelled thymus cells were traced to the liver and spleen of the receptor, while labelled lymph gland cells were traced to the liver and bone marrow. In a later experiment with a different technique the lymph gland cells were also traced to the spleen, but not to the same extent as the thymus cells (Diderholm & Fichtelius 1959). The purpose of the following experiment was to demonstrate *in vitro* a similar difference between thymus and lymph gland cells.

#### METHOD

Homogenates of spleen, liver and bone marrow (equal parts of the organs and physiological saline or distilled water) were prepared as follows:

Thymus suspension and lymph gland suspension were prepared as follows. The thymus and lymph glands from a guinea pig were roughly freed of connective

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By

## THE FIGHTFLIES

Received 27.1.64

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BEFORE AND AFTER THE PASSAGE OF GLASS BEADS  
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### Experiment 1 A

In experiments involving the transfusion of  $P^{32}$ -labelled thymus cells and lymph gland cells Fichtelius (1958 a and b) demonstrated differences between the two types of lymphocytes. Labelled thymus cells were traced to the liver on the 1st day after transfusion, whereas lymph gland cells were not traced to the same extent as the thymus cells (Diderholm & Fichtelius 1959). The purpose of the following experiment was to demonstrate *in vitro* a similar difference between thymus and lymph gland cells.

## METHOD

[illegible]

Thymus and lymph glands from a guinea pig were roughly freed of connective

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tissue washed in Earl's solution their surfaces were carefully freed of remaining connective tissue and they were then washed again in Earl's solution. The organs were then cut up finely and placed into a glass vessel. 5 ml of Earl's solution were added and the pieces of tissue were stirred vigorously with a forceps or scissor blades and pressed against the sides of the glass vessel. The tissue mixture was then sieved through ordinary muslin.

2 ml of the thymus suspension and lymph gland suspension respectively were poured into each of the burettes with spleen, liver and bone marrow beads and were allowed to pass through 55 cc of these, after which the tap was closed. 1 minute after the start the tap was opened and the suspension was allowed to pass through a further 0.5 cc of beads after which the tap was closed again. Every succeeding minute the suspension was passed through 0.5 cc of 'new' beads in this way. At 5 minutes after the start the suspension was let down to the 10 cc mark and at 10 minutes it was released through the tap and the first three drops taken for examination. The number of cells per cmm in the suspension before and after its passage through the beads was then counted (dilution 1:20 or 1:100  $\times 32$  B squares counted in the Burkner chamber). The number per cmm after the passage was given as a percentage of the number per cmm before. Three thymus suspensions and three lymph gland suspensions were passed through glass beads coated with saline extracts, and four suspensions of each kind through glass beads coated with water extracts.

## RESULTS

As can be seen in Table 1, there were fewer cells per cmm after passage through spleen and bone marrow beads. On the other hand there were more cells per cmm after passage through liver beads. No significant difference was observed between the thymus and lymph gland cells.

TABLE 1

*No. of Cells per cmm after the Passage through Glass Beads Coated with Extract as a Percentage of the Number per cmm before Passage*

	Spleen beads	Liver beads	Bone marrow beads
<i>Saline extract</i>			
Thymus suspension	90.2	96.3	72.0
	103.9	182.4	68.6
	76.4	156.9	44.4
Mean	90.2	145.2	61.7
Lymph gland suspension	88.2	163.2	111.8
	93.2	106.8	50.0
	101.7	230.5	74.6
Mean	94.4	166.8	78.8
<i>Water extract</i>			
Thymus suspension	48.9	118.9	91.1
	56.1	129.0	61.3
	61.9	228.6	66.6
	88.2	202.0	80.4
Mean	63.8	169.6	74.9
Lymph gland suspension	44.9	146.9	67.3
	45.5	276.4	36.4
	51.3	200.0	74.4
	58.3	291.7	83.3
Mean	50.0	218.8	65.4

The study confirms earlier observations that these ruptures occur independent of age and pathologic changes of the aorta. Middle aged men form the largest group probably because they most often expose themselves to serious traffic accidents. In contrast to earlier observations the material contains more motorists than pedestrians. This is probably because most accidents happened on country roads where as civilization proceeds pedestrians are a vanishing race.

Traumatic rupture of the aorta is usually accompanied by fatal intrathoracic haemorrhage. The survival time is only in a few instances long enough to permit surgical treatment. In a number of cases this injury is the single serious lesion in cases of traffic accidents. There are therefore ample reasons to analyse, as far as possible, the mechanical forces acting in these instances in order to find adequate counter measures. Such analysis is the subject of another study (Lundvall 1964).

#### SUMMARY

Twenty one cases of fatal traumatic ruptures of aorta are reported. All cases except one were traffic accidents. Middle aged men were the largest group of the injured. The ruptures were predominantly located at the isthmus of aorta. In 16 cases only one rupture occurred, in 5 cases there were 2 or more ruptures. Severe intrathoracic haemorrhage was present in all cases and probably represented the single cause of death in 5 cases.

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## THE MECHANISM OF TRAUMATIC RUPTURE OF THE AORTA

By

JON LUNDEVALL

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Traumatic rupture of the aorta may occur when the body, or the upper part of the body, is exposed to a sudden change of velocity by blunt force. The ruptures usually occur in the isthmus region, more rarely in the ascending part of aorta, and seldom in other parts of the vessel. The traumatic ruptures are nearly always transverse. (For references, see Lundevall 1964)

The detailed mechanism which produces these ruptures have been discussed for many years, and opinions have in part been contradictory. The aim of this study is to give a survey of the different views, and to contribute to the discussion by personal observations.

### *Earlier Investigations*

Rindfleisch (1893) held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching.

Oppenheim (1918) ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg. By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves. In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point. In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg. Dissecting aneurysms frequently occurred during the experiment. In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents. Oppenheim admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death. The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered.

Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow.

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents. Even Kleinsasser (1943), Strassmann (1947) and Tannenbaum & Ferguson (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures.

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body.

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture. Letterer (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart's downward traction on the aorta.

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest. and Kleinsasser (1943) sustained this opinion.

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture. Tannenbaum & Ferguson (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance those cases in which rupture is caused by flying pieces of wood or stone, hitting the chest.

Marshall (1938) pointed out that when the body is decelerated during forward motion, the hilus of the left lung will exert a traction on the parietal pleura covering the aorta. By this mechanism aorta is probably kinked in such a way that rupture occurs.

Cammack *et al* (1939) found that antero-posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart.

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region.

To summarize, earlier authors agree that traumatic rupture occurs in connection with a considerable and sudden change of velocity of the body. The direction of the velocity change may vary from one case to another and is often but not always accompanied by a contusion of the chest. There is no agreement why the isthmus region is most exposed to ruptures.

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-



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### *Earlier Investigations*

*Rindfleisch (1893)* held the opinion that traumatic rupture is produced by a sudden stretching of the vessel, and that the upper thoracic portion of aorta is most exposed to such stretching.

*Oppenheim (1918)* ligated the branches of human aortas and filled the aortas with water at a pressure up to about 3000 mm Hg. By this procedure, ruptures regularly occurred in the ascending part just above the semilunar valves. In one instance there was a rupture at isthmus at 790 mm Hg pressure, but this particular aorta was calcified at this point. In one perfectly healthy aorta two ruptures occurred, one just above the semilunar valves and one at isthmus, at a pressure of 2070 mm Hg. Dissecting aneurysms frequently occurred during the experiment. In two respects therefore, the ruptures caused by this procedure differed from those commonly observed during actual accidents. *Oppenheim* admitted that a rise of the blood pressure to such high values hardly ever occurs in living persons, but he surmised that considerably lower internal pressures may cause rupture *in vivo* because the resistance of aorta against stress then is lower than after death. The reason for this, he thought, was that tissue fluidization ceases at death, and that the body temperature is lowered.

Oppenheim did not state at which rate the pressures were applied, but it is inferred from his remarks that this was rather slow

Klotz & Simpson (1932) found in similar experiments that aorta from young persons resisted an internal pressure of 1000 mm Hg without rupturing, but they nevertheless assumed that it is the sudden rise of the blood pressure which ruptures the aorta during accidents. Even Kleinsasser (1943), Strassmann (1947) and Tannenbaum & Ferguson (1948) were of the opinion that a rise in blood pressure is an important factor for the occurrence of these ruptures

No experiments have been reported in which the blood pressure in the thoracic portion of aorta has been measured during impacts to the body

A number of authors have stressed the importance of aorta's topography in order to explain the mechanism of rupture. Letterer (1924) thought that in cases where the individual fell on his feet or buttocks from a height, the rupture was caused by the heart's downward traction on the aorta

Shennan (1929) held the opinion that the relative fixation of the aorta to its surroundings at isthmus had a "hinge" effect, causing rupture at this point by sudden movements of the chest, and Kleinsasser (1943) sustained this opinion

Hass (1944) more generally stated that any rapid relative displacement of two adjacent parts of the body causes a stress at the junction, and that this is the basis for the mechanism of aortic rupture. Tannenbaum & Ferguson (1948) agreed to some extent, but said that some cases of traumatic rupture of the aorta could not be explained by Hass' theory, as for instance those cases in which rupture is caused by flying pieces of wood, or stone, hitting the chest

Marshall (1938) pointed out that when the body is decelerated during forward motion, the hilus of the left lung will exert a traction on the parietal pleura covering the aorta. By this mechanism aorta is probably kinked in such a way that rupture occurs

Canimack *et al* (1959) found that anterior posterior compression of the chest will cause a dislocation of the heart to the left, and a torsion of the aorta with a maximal stress on its wall near the base of the heart

Zehnder (1960) deduced from theoretical considerations that the main factor for different traumata is a hyperflexion of the aortic arch, with a predominant stress on the isthmus region

To summarize, earlier authors agree that traumatic rupture occurs in connection with a considerable and sudden change of velocity of the body. The direction of the velocity change may vary from one case to another, and is often but not always accompanied by a contusion of the chest. There is no agreement why the isthmus region is most exposed to ruptures

A discussion of the mechanism of traumatic rupture of the aorta must be based on an assessment of the forces acting on the aorta dur-

ing velocity changes, and the ability of the aortic wall to resist these forces. The following observations were made in order to obtain some data for this discussion.

## MATERIAL

Aortas from 10 bodies were studied. The bodies were of 7 men and 3 women aged between 35 and 84 years. Nine of the patients died of disease, one (the youngest) of barbiturate poisoning. All aortas presented various degrees of atherosclerosis but none had calcifications of the aortic wall. The observations were made 1-2 days after death.

### Anatomical Observations

## RESULTS

The course of the aortic arch deviated 20-25° from the sagittal plane. In each case, the thoracic part of aorta was relatively mobile, but the isthmus region was slightly more fixated to its surroundings than the portions of aorta above and below. The great arterial branches from the arch did not particularly restrain aorta's movements, nor did the intercostal arteries. The arterial branches were easily stretched. The recurrent nerve, running close to the concavity of the aortic arch at isthmus, was fairly resistant to traction. In some instances the nerve was readily palpable through the aortic wall when aorta was pressed against the pulmonary root.

The heart was relatively resistant against vertical movements, but was somewhat more mobile transversally, and could also to some extent be rotated along its long axis.

The root of the left lung was relatively well fixed against any movements. The anatomical measures of the aortas are given in Table 1.

TABLE 1  
*Anatomical Measures of Aorta from 10 Bodies*

Body			Arch diam cm	Aorta's radius cm		Thickness of wall cm	
No	Age (years)	Sex		p aortic	isthmus	p aortic	isthmus
I	35	♂	8	0.9	0.7	0.125	0.12
II	36	♂	6	1.0	0.8	0.155	0.165
III	39	♀	8	0.7	0.6	0.18	0.155
IV	47	♂	11.5	1.2	0.9	0.25	0.18
V	64	♂	10	1.4	1.1	0.215	0.2
VI	64	♀	8	1.3	1.0	0.14	0.14
VII	68	♂	10	0.9	0.6	0.18	0.13
VIII	79	♂	11	1.2	1.0	0.225	0.185
IX	80	♀	9	1.0	0.8	0.20	
X	84	♂	7.5	1.4	1.2	0.18	0.115
Mean values			8.9	1.1	0.87	0.185	0.167

Arch diameter is the horizontal distance between the outer (medial) wall of p aortic and the outer (lateral) wall in the isthmus region at the level of the top of the concave wall of the arch.

Aorta's radius at p aortic and isthmus was calculated from the measured circumference.



Fig 1

Dumbbell specimen

### Observations on the Resistant Power of the Aortic Wall

Specimens of standardized measures (Dumbbell specimens, Fig 1) were punched from each of the 10 aortas already described

From each aorta, four specimens (Nos 1-4) were taken from the p a se, with their long axis along the blood stream, and symmetrically distributed around aorta's circumference. The next four specimens (Nos 5-8) were from the isthmus region, and were also in symmetrical positions around the circumference, their long axis parallel to the blood stream. The last two specimens (Nos 9-10) were from the posterior and the lateral parts of aorta's wall a little below isthmus, and were also taken from the lower thoracic portion of each of 2 aortas. In each case, one specimen was longitudinal, the other transversal to the axis of the vessel, at the same level.

Adventitia was removed from the specimens, and the thickness of the wall was measured with a micrometer in two specimens from each aorta, one from p a se, and one from the isthmus region.

The specimens were stretched until they ruptured in an Instron stretching machine, kindly placed at disposal by the Norwegian Institute for Industrial Research. The rate of stretching was the machine's fastest, 50 cm per minute. The load at the moment of rupture was recorded. The results are summarized in Table 2.

TABLE 2  
The Tensile Strength of the Wall of Aortas from 10 Bodies  
Load (grams) of Dumbbell Specimens at Moment of Rupture

Body No	Spec no (p a se)				Spec no (isthmus)				Spec no (p desc)		Lower (p desc)	
	1	2	3	4	5	6	7	8	9	10	Long	Tr
I	530	490	730	400	490	420	360	190	420	320	410	890
II	375	730	820	455	580	215		215				
III	840	690	720	800			370	335	400	320		
IV	525	460	550	590	555	365	430	265	430	455		
V	565	440	540			485	235	200		240		
VI	690		540	490	300	200	360	310	315	360		
VII	675	440	490	560	530	430	390	320		650		
VIII		490	575	560		310	380	280				
IX	590	350	335	625		640	440	315	495	380	280	595
X	370	300	620	520	280	190	120	220	400	245		
Mean values		517.8				300.8				397.4		

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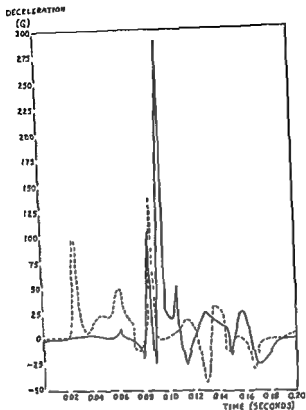


Fig 2

Deceleration/Time diagram (From Odelgard &amp; Weman 1957)

----- Automobile      ——— Shoulders

There are three kinds of mechanical forces which may act on the aortic wall during rapid velocity changes:

A Traction and pressure on aorta by other thoracic organs, and torsion of the aorta

B Strain waves in the aortic wall

C Pressure variations in aorta's blood content

These forces will be discussed on the basis of the observations referred, and other author's observations

A *The effect of traction and pressure on aorta by other thoracic organs and torsion of the aorta* From the anatomical observations it appears that during sudden changes of velocity in the sagittal plane (as in the example studied by Odelgard & Weman 1957) both the arch and the lower thoracic portion of the descending aorta are probably dislocated forwards or backwards in relation to the more fixed areas at isthmus and the base of the heart. At these two points therefore,

During the tests, a few specimens were crushed at the fixation points, and were omitted as indicated in the table

During the slow stretching, the specimens were considerably elongated and reduced in thickness before they ruptured, and did not regain their original thickness when the stretching was discontinued. The resistance of the specimens against the stretching was therefore mainly due to viscosity, not to the elasticity of the tissue.

The resistant property of the wall varied considerably around the circumference at each level within each aorta. However, the resistance was mainly less at isthmus than in the ascending and descending parts, and the transversal specimens resisted twice the load which caused rupture of the longitudinal specimens.

## DISCUSSION

### 1 Forces Acting on the Aortic Wall

A typical accident in which rupture may occur is the case of a motor car driver who is thrown against the steering wheel as the car collides frontally. This situation has been studied experimentally by Odelgard & Weman (1957). In their experiment a dummy of anthropometric measures was rigged in the driver's seat of a car with a safety harness. The car was dropped vertically and hit the ground at a speed of 60 km/h. The movements of the car and the dummy were recorded, and derivations of the recordings gave, among others, the diagram shown in Fig. 2.

The dummy's shoulders were subjected to heavy vibrations lasting for about 0.12 seconds, during which decelerations up to 290 G occurred (G expresses the degree of change in velocity in multiples of magnitude of the acceleration of gravity). Other recordings showed that the hips of the dummy were also vibrated but not synchronous with the shoulders. The authors pointed out that, owing to the elastic response of the individual's body and the car's wheel, the magnitude of body vibrations on impact is difficult to predict. It is reasonable, however, to assume that the absence of a damping harness will accentuate the peaks of deceleration, and also that these peaks will rise with the collision speed.

The internal organs, including the aorta, will presumably also be exposed to vibrations during an impact. The magnitude of these vibrations, however, is unknown at present because no experimental studies have been reported. Some authors referred by Zehnder (1960) have made quantitative calculations of the degree of deceleration or acceleration necessary to produce rupture. These calculations are based on the assumption that the change of velocity of the body, and of the aorta, is steady during an impact, but Odelgard & Weman's experiments have shown that this probably is not the case.

authors already referred have stated that a sudden increase of the blood pressure is an important factor in the mechanism of traumatic rupture of the aorta no direct observations in this pressure have been reported. The discussion of this factor must therefore be based on theoretical considerations.

A local rise in the blood pressure may be caused in two ways. Firstly by a local compression of aorta followed by displacement of the blood with an expansion of the adjacent uncompressed parts of aorta. Secondly by the propagation of a pressure wave in the blood content causing a waterhammer-effect.

The first of these mechanisms is contingent upon the compression being applied for a time long enough to allow the blood to flow away from the compressed area and the rate of application of the pressure being so fast that only a limited area of the aortic wall at both sides of the compression is expanded. Moritz (1932) found that pressures of 800-1000 mm Hg applied in the aorta of living rats caused rupture of aorta or its branches. Aorta did not rupture because the blood was led away too fast. It is difficult to imagine that this mechanism of blood pressure increase can produce a single circular rupture as is usually seen in accident cases.

The second mechanism is illustrated by the following example. If a person during forward movement is suddenly stopped the blood in the aortic arch will continue forwards by its inertia and exert a pressure against the anterior wall of the ascending part of aorta and a corresponding traction on the posterior wall in the isthmus region. The pressure is proportional to the weight of a horizontal column of blood lying close to the concave inner surface at the top of the arch and which has a base of 1 sq cm. From the mean of measures of 10 aortas referred in Table 1 and the blood's specific weight it was calculated that this column weighs 9.4 g. At a given degree of velocity change expressed in G's the pressure is therefore 9.4 G g/sq cm.

According to Wehn (1963) the resulting tension at different points of the aortic wall can be calculated in the following way.

The aortic arch can be regarded as the half of a ring formed tube as shown in Fig. 3.

The ring's axis is the line AB lying in the paper's plane. Each

on it

of re

curve

distance in the point E is found by tracing a diameter through this point and prolong it to the axis AB. R is the distance CF and its plane of rotation is vertical to the paper's plane.

Corresponding to the line FG the radius of curvature (R) shifts from  $+\infty$  to  $-\infty$  which means that R referring to points on the concave side of the tube's surface has a negative sign. The point D therefore has a radius of curvature (R) equal to  $-CD$ .

The formula for circular (meridional) stress ( $I_1$ ) is (Wehn 1963)



local longitudinal stretchings of the aortic wall occur. In cases in which the antero-posterior diameter of the chest is reduced (as when the chest is pressed against the steering wheel), the heart will probably be dislocated to some extent to the left and also be rotated along its long axis. A tangential torsion stress will then act on the aorta, preferentially on the part just above the semilunar valves, and tend to produce a spiral formed rupture.

During velocity changes along the vertical axis of the body (as when the individual falls on his feet or buttocks), the arch and the descending part of aorta will probably be dislocated downwards in relation to the isthmus region, producing a stretching of the upper lateral part of the wall at isthmus. The arch will then be pressed against the left hilus, and the recurrent nerve may exercise a cutting pressure from below.

In traffic accidents, abdominal injuries often occur, causing an increased intra-abdominal pressure. The diaphragm is often ruptured, and abdominal viscera are occasionally displaced into the left thoracic cavity. The left lung's hilus is then pressed upwards, resulting in an increased bending, or even kinking of the aortic arch. This mechanism will therefore tend to produce a transverse rupture in the isthmus region.

The cervical vessels are stretched when the head is moved backwards, or when the aortic arch is pressed downwards. Even if these vessels are easily stretched, they may exert a local traction on the aortic arch.

In most accidents, the movements of the body are probably complex, the directions of velocity changes varying during fractions of a second. Particularly in cases in which a pedestrian is hit by a car, he often tumbles head over heels, and it is virtually impossible to reconstruct which of the stresses discussed is the predominating in the mechanism causing aortic rupture. It will also be apparent that the magnitude of the forces at present is incalculable.

*B Strain waves in the aortic wall* Aldman (1962) has shown that when an elastic belt is stretched, a strain wave, which is a local elongation of the material, will start at one anchoring point of the belt. The wave travels along the material and is reflected at the belt's ends. If the belt is stretched faster than the material can be elongated, the belt will rupture. The lowest velocity at which rupture occurs by this effect is called the critical velocity, which can be estimated when the velocity of the strain waves are known.

It may reasonably be assumed that similar strain waves occur in the elastic wall of aorta in accidents where a part of aorta is suddenly stretched. As no experiments have been made to estimate this condition, the importance of the phenomenon for the causation of rupture of the aorta is still unknown.

*C Pressure variations in aorta's blood content* Although several

The formula for longitudinal stress in a straight tube is (Windsor 1959)

$$L_s = \frac{p r}{2} \text{ g/cm}$$

and the corresponding tension (S) is

$$S_s = \frac{p r}{2 h} \text{ g sq cm}$$

Wehn (1963) has calculated that this tension has the same value in a curved tube (the hoop tension) as in a straight one and the tension therefore is independent of R

The numerical values for S at different points were calculated from the data already referred and were found to be

$$\begin{aligned} S \text{ at isthmus} & 300 \pm 20 \text{ G g sq cm} \\ S \text{ at pulse} & 400 \pm 28 \text{ G g sq cm} \end{aligned}$$

In the outlined situation the waterhammer effect will therefore cause a tension in the aortic wall which is greater in pulse than in the isthmus region and the longitudinal tension (which would produce a transverse rupture) is about half as great as the circular tension

### 3 The Resistant Power of the Aortic Wall

Zehnder (1960) who measured the traction tolerance of specimens from aortas from 97 bodies found that the tolerance varied considerably among individuals and also within each single aorta. The tolerance was generally less against traction along the blood stream than against transverse (circular) traction. Although the rate of onset of traction was not stated the author concluded that the percentage elongation of the specimens before they ruptured expressed the elasticity of the aortic wall.

In ideal experiments the resistant power of the aortic wall should be tested during conditions similar to those occurring in actual accidents. The tests should therefore be done on living tissue and stress should be applied with a rate of onset and a duration as would occur in an accident. This was not feasible during laboratory conditions and for this reason a method similar to Zehnder's was employed.

In the experiments referred only the resistance due to viscosity of the tissue was measured. During actual accidents the stress on the aortic probably sets in so rapidly that there is no deformation of the wall before rupture occurs. A resistance due to viscosity is then excluded and the experiments do only coarsely reflect the resistant power of the aortic wall during such accidents.

$$L_1 = p r \left( 1 - \frac{r}{2 R} \right) \text{ g/cm}$$

where  $p$  is the pressure acting on the wall from the inside. On the convex side  $L_1$  is less than  $p r$ , while on the concave side  $L_1$  is greater than  $p r$ . The circular stress has a maximum on the concave side of the tube's wall and a minimum on the convex side.

The tension on the wall ( $S_1$ ) produced by the stress ( $L_1$ ) is defined as the stress divided by the wall's thickness ( $h$ ).

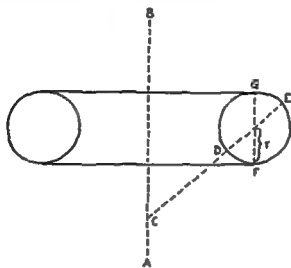


Fig 3

The aorta as a curved tube

For numerical evaluation of the circular tension caused by the waterhammer-effect in the mechanism outlined above, the mean values of  $R$ ,  $r$  and  $h$  in Table 1 were used, although  $R$  and  $r$  probably are greater *in vivo* than post mortem, because of a possible postmortal contraction of aorta. The pressure  $p$  is the sum of the physiological blood pressure (about 100 mm Hg or 136 g/sq cm) and the additional pressure caused by the waterhammer-effect, 9.4 G g/sq cm.

From the formula

$$S = \frac{p r}{h} \left( 1 - \frac{r}{2 R} \right)$$

the following values were found

$S_{1 \text{ max}}$	at concave side of isthmus	811 + 56 G g/sq cm
$S_{1 \text{ min}}$	at convex side of isthmus	639 + 44 G g/sq cm
$S_{1 \text{ max}}$	at concave side of p asc	956 + 66 G g/sq cm
$S_{1 \text{ min}}$	at convex side of p asc	709 + 49 G g/sq cm

region. From the study of *Odelgard & Weman* (1957) we know that, in an autocar collision, the driver's shoulders and hips undergo velocity changes of similar magnitude as those calculated above. It is reasonable to assume that also the aorta may be exposed to velocity changes of such strength that a waterhammer effect is of importance in the mechanism causing rupture.

The waterhammer-effect alone would theoretically cause circular and longitudinal ruptures at about equal degrees of velocity change. *Schnurbein* (1926) found that when increased internal pressure was applied in the straight portion of the descending aorta in rabbits, the ruptures which occurred were longitudinal, not transverse. Why then, are the ruptures occurring in aorta in actual accidents nearly always transverse? The reason probably is that the other forces already discussed, which tend to cause transverse ruptures, act together with the waterhammer mechanism.

A study of the mechanism of traumatic rupture of the aorta is necessary for the choice of adequate countermeasures. There is probably not much we can do to protect the pedestrian when he is hit by a fast-moving car, but the motorist driving a car which collides can be protected to a certain degree against this serious injury. The aim of this protection must be to reduce the rate of deceleration, and the development of adequate devices should be encouraged. The ideal design of such devices depends on a precise knowledge of the mechanism which causes the damage.

#### SUMMARY

The mechanism causing traumatic rupture of the aorta is discussed on the basis of earlier observations and personal studies. The basis for the discussion is at present, however, insufficient, and the conclusions must be drawn with caution.

Of the different forces acting on the aortic wall, only the effect of a pressure wave in aorta's blood content (a waterhammer-mechanism) could be approximately calculated.

Then tensile strength of the aortic wall of human bodies was tested under static conditions. The results indicated that the isthmus region is less resistant to stress than the ascending and descending parts of aorta.

On comparing the results it is concluded that rupture of the aorta is probably caused by the effect of forces of different kinds. A waterhammer effect probably plays an important rôle, but local pressure and traction and strain waves in the wall act simultaneously and explain why the ruptures are nearly always transverse, and preferentially located in the isthmus region.

Secondly, dead tissue probably has a higher power of resistance than living tissue (*Oppenheim* 1918 and personal observations) *Schnurbein* (1926) found that aortas of rabbits ruptured at about 1.3 atm internal pressure, whether the rabbits were freshly killed or already had a well developed rigor. All his animals, however, were dead at the time of the experiments.

These objections mainly concern the absolute values of resistance. The measures probably give an approximately reliable picture of the relative resistant property of the different areas of each single aorta. A calculation of the resistant power of the different parts of aorta, based on the mean values from Table 2, seems therefore justified.

By derivation of the results it was found that the main resistant power of a cross section of the aortic wall against longitudinal traction is

at isthmus	5250 g/sq cm
at p asc	7400 g/sq cm

It was therefore concluded that the tissue of the aortic wall is somewhat less resistant to slow traction in the isthmus region than in p asc. It was also presumed that the resistant power against transversal traction is the double of these figures in the respective areas, although this was measured in only two of the aortas.

*Comparison between damaging forces and the resistant power of the aortic wall.* From the anatomical observations it appears that traction and pressure on aorta by other thoracic organs, torsion of the aorta and strain waves in the aortic wall during sudden stretching will mainly tend to produce transverse ruptures. The observations indicate that the ruptures caused by these forces preferentially would occur in the isthmus region, but also in some case in the ascending part of aorta.

In a certain situation, commonly occurring during accidents leading to rupture of the aorta, a waterhammer-effect caused by a pressure wave in the blood of the aortic arch must also be taken into consideration. The amount of this effect rises with the degree of velocity change, and by derivation of the mean of the experimental results it was found that the waterhammer-effect alone would produce rupture of the aortic wall at the following points, and at the following degrees of velocity change.

Transverse rupture at isthmus	at 196 G
Longitudinal rupture at concave side of isthmus	173 G
Longitudinal rupture at convex side of isthmus	224 G
Transverse rupture in p asc	250 G
Longitudinal rupture at concave side of p asc	210 G
Longitudinal rupture at convex side of p asc	286 G

Although the figures are based on observations subject to criticism, it is reasonable to conclude that a somewhat higher degree of velocity change is necessary to produce rupture in p asc than in the isthmus.

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## A FACTOR IN THE LIVER SCATTERING AGGREGATES OF LYMPHOID CELLS IN ARTIFICIALLY PREPARED SUSPENSIONS

By

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Received 27 II 64

It was by chance observed that the number of cells in suspensions of thymus and lymph gland cells increased on the addition of liver extract. Regardless of the type of liver factor concerned here, this observation sheds some light on the possible errors that may arise if the number of cells is calculated in artificial suspensions.

### THE NUMBER OF SUSPENDED LYMPHOID CELLS BEFORE AND AFTER THE PASSAGE OF GLASS BEADS COATED WITH ORGAN EXTRACTS

#### Experiment 1 A

In experiments involving the transfusion of  $P^{32}$ labelled thymus cells and lymph gland cells, Fichtelius (1958 a and b) demonstrated differences between the two types of lymphocytes. Labelled thymus cells were traced to the liver and spleen of the receptor, while labelled lymph gland cells were traced to the liver and bone marrow. In a later experiment with a different technique the lymph gland cells were also traced to the spleen, but not to the same extent as the thymus cells (Diderholm & Fichtelius 1959). The purpose of the following experiment was to demonstrate *in vitro* a similar difference between thymus and lymph gland cells.

#### METHOD

Homogenates of spleen, liver and bone marrow (equal parts of the organs and physiological saline or distilled water) were prepared from guinea pigs weighing approximately 200 g. The homogenates were centrifuged at 400 g for 10 min and the supernatant fluid was used for the experiments.

Thymus suspension and lymph gland suspension were prepared as follows. The thymus and lymph glands from a guinea pig were roughly freed of connective

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### Experiment 1 B

In experiment 1 A the change in the number of cells per cmm as a result of passage through glass beads coated with extract was investigated, in experiment 1 B the technique was modified so that the total number entering and leaving the column could be counted

### METHOD

The preparation of suspensions and beads coated with extract was carried out as in experiment 1 A. The extract was prepared with distilled water. When at 10 minutes after the start the suspensions were allowed to run out through the tap 2 ml of Earl's solution were added. The tap was left open for the following 5 minutes. The fluid collected was measured carefully. The total number of cells that were introduced among the beads and the number collected after passage through them were counted with the aid of the Burkner chamber. The number that passed through was given as a percentage of those allowed to come in. 9 separations were performed with thymus suspensions and 9 with lymph gland suspensions.

### RESULTS

As may be seen in Table 2, between 50 and 70 per cent of the cells passed through the spleen and bone marrow beads. No difference was observed between the thymus and lymph gland cells. On the other hand there were more cells in the fluid collected after the passage through the liver beads than in the 2 ml of suspension that was poured in. The thymus and lymph gland cells behaved similarly in this respect.

TABLE 2

*The Passage of Cells through Glass Beads coated with Extracts given as Percentages*

	Spleen beads	Liver beads	Bone marrow beads
Thymus suspension	57.5 81.4 117.3 30.6 — 117.3 63.1 30.4 58.6	147.4 151.0 154.6 85.5 139.3 155.0 98.9 208.9 100.0	17.8 60.0 59.8 77.8 51.6 111.0 45.4 40.0 62.6
Mean	72.8	137.8	58.5
Lymph gland suspension	71.8 39.8 51.0 141.9 77.2 93.7 54.9 59.2 32.9	153.7 149.3 153.2 138.0 140.7 129.4 126.8 268.9 77.6	36.2 43.3 38.1 118.8 54.1 28.6 57.0 49.2 48.7
Mean	69.6	148.6	52.7



tissue washed in Earl's solution their surfaces were carefully freed of remaining connective tissue and they were then washed again in Earl's solution. The organs were then cut up finely and placed into a glass vessel. 6 ml of Earl's solution were added and the pieces of tissue were stirred vigorously with a forceps or scissor blades and pressed against the sides of the glass vessel. The tissue mixture was then sieved through ordinary muslin.

1 ml of the thymus suspension and lymph gland suspension respectively were poured into each of the burettes with spleen liver and bone marrow beads and were allowed to pass through 55 cc of these after which the tap was closed. 1 minute after the start the tap was opened and the suspension was allowed to pass through a further 05 cc of beads, after which the tap was closed again. Every succeeding minute the suspension was passed through 05 cc of 'new' beads in this way. At 9 minutes after the start the suspension was let down to the 10 cc mark and at 10 minutes it was released through the tap and the first three drops taken for examination. The number of cells per cmm in the suspension before and after its passage through the beads was then counted (dilution 1:20 or 1:100  $2 \times 32 B$  squares counted in the Burker chamber). The number per cmm after the passage was given as a percentage of the number per cmm before. Three thymus suspensions and three lymph gland suspensions were passed through glass beads coated with saline extracts and four suspensions of each kind through glass beads coated with water extracts.

## RESULTS

As can be seen in Table 1, there were fewer cells per cmm after passage through spleen and bone marrow beads. On the other hand there were more cells per cmm after passage through liver beads. No significant difference were observed between the thymus and lymph gland cells.

TABLE 1

No. of Cells per cmm after the Passage through Glass Beads Coated with Extract as a Percentage of the Number per cmm before Passage

	Spleen beads	Liver beads	Bone marrow beads
<i>Saline extract</i>			
Thymus suspension	90.2 103.9 76.4	96.3 182.4 156.9	72.0 68.6 44.4
Mean	90.2	145.2	61.7
Lymph gland suspension	88.2 93.2 101.7	167.2 106.8 230.5	111.8 50.0 74.6
Mean	94.4	166.8	78.8
<i>Water extract</i>			
Thymus suspension	48.9 56.1 61.9 88.2	118.9 129.1 228.1 202.0	91.1 61.3 66.6 80.4
Mean	63.8	169.6	74.9
Lymph gland suspension	44.9 45.5 51.3 58.3	146.9 236.4 200.1 291.7	67.3 36.4 74.4 83.3
Mean	50.0	218.8	65.4

## Experiment 2 B

Assume that aggregates occur in the cell suspensions and that these aggregates are of such an order of magnitude that a sample from the suspension taken with a fine pipette will contain fewer cells per cmm than a sample taken with a coarser pipette. In this case the effect of the liver factor would be less noticeable if coarser pipettes were used for the taking of the samples. In the following experiment which is a repetition of experiment 2 A pipettes of different sizes were used.

## METHOD

0.2 ml of cell suspension was mixed with 0.2 ml of Earl's solution or with 0.02 ml of water extract of liver. The number of cells per cmm was then counted in a Burkner chamber after diluting 1:20 with Earl's solution. The samples were taken 5 minutes after mixing both with so called blood pipettes of 0.025 cmm and with coarser pipettes of 0.05 or 0.1 cmm.

## RESULTS

Table 4 shows that in all cases a higher number of cells per cmm was obtained in the mixture with Earl's solution when the sample was taken with a coarser pipette. Further the effect of the liver factor appeared to be less in all cases where a coarse pipette was used than in cases where finer ones were used. An attempt at making a direct estimate of the aggregation tendency failed since in all mixtures this was very insignificant.

TABLE 4

*The Number of Cells per cmm after the Addition of Earl's Solution and of Liver Extract using Pipettes of Different Sizes for the Sampling*

	0.025 + 0.1		0.025 + 0.300
A Thymus suspension + Earl's solution	4600	<	9400
B Thymus suspension + liver extract	19300		16300
B/A × 100	420	>	173
A Lymph gland suspension + Earl's solution	10300	<	12800
B Lymph gland suspension + liver extract	18600		18800
B/A × 100	181	>	147
	0.025 + 0.1		0.100 + 1.900
A Thymus suspension + Earl's solution	1350	<	2300
B Thymus suspension + liver extract	3700		3900
B/A × 100	274	>	166
A Lymph gland suspension + Earl's solution	2150	<	3700
B Lymph gland suspension + liver extract	4500		5600
B/A × 100	209	>	149

The error of the method in all of these investigations is very large, since relatively few cells were counted in the Burkner counting chamber. The coefficient of variation on counting  $2 \times 32$  B squares from different samples of the same suspension is approximately 10 per cent. The

When Earl's solution alone was passed through the liver beads in the same way as the cell suspensions, no cells were found in the fluid collected. Neither did direct examination of the liver extract reveal any cells. Thus it must be concluded that only thymus or lymph gland cells were counted after passage.

It is theoretically possible that a true increase in the number of cells occurs, but it is more probable that this is only apparent. The small number of cell aggregates, as a rule comprising fewer than 10 cells, which was observed in the suspensions on careful examination, may have been dispersed by some factor in the liver extract, so that on counting in the Burkner chamber more cells were recorded than before the passage through the liver beads.

### THE INFLUENCE OF LIVER EXTRACT ON THE NUMBER OF SUSPENDED LYMPHOID CELLS CONVENTIONALLY COUNTED

#### *Experiment 2 A.*

The following experiment was carried out in order to preclude, if possible, a true cell increase as an explanation for the results.

#### METHOD

0.5 ml of Earl's solution was mixed with 0.5 ml of cell suspension (thymus and lymph gland suspensions prepared as in experiment 1). 0.5 ml of the same suspension was mixed with 0.5 ml of a water extract of liver. The number of cells per cmm was then counted in a Burkner chamber (dilution 1:20  $2 \times 32$  B squares counted), both immediately after and also 20 minutes after mixing.

#### RESULTS

It may be seen in Table 3 that the number of cells per cmm after the addition of liver extract was more than 200 per cent of the number per cmm after the addition of Earl's solution. This effect of the liver extract occurred immediately. When the liver factor was added under the microscope no changes were noted among the cells. Further examination of the liver extract revealed no cells. The conclusion must therefore be drawn that the cell increase after the addition of liver extract was apparent.

TABLE 3

*No. of Cells per cmm after the Addition of Earl's Solution and of Liver Extract*

	Immediately	After 20 mins
A Thymus suspension + Earl's solution	8600	11300
B Thymus suspension + liver extract	30000	26200
A Lymph gland suspension + Earl's solution	3500	2200
B Lymph gland suspension + liver extract	10100	11500

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# HISTOCHEMICAL OBSERVATIONS ON WOUND HEALING IN DENERVATED AND HEALTHY RAT SKIN

By

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Received 13 III 64

Wound healing has been shown to be distinctly slower in denervated rat skin than in a healthy animal (Elfving 1959). Attempts to outline the pathogenesis of this retardation would be important both pathologically and surgically.

In several previous investigations (Raekallio 1960, 1961, 1963 a, b, Raekallio & Ievonen 1963 a, b, c, Raekallio 1964, Raekallio & Seligman 1964) histochemical methods for enzymes have been demonstrated to provide a tool for detecting some of the very first reactions in healing wounds. The differential enzymatic response to injury in denervated and healthy skin is the particular object of this investigation.

## MATERIAL AND METHODS

12 healthy four month old Sprague Dawley rats of approximately 200-250 g weight were used. All the animals were kept on standard laboratory diet and water.

Two weeks preoperatively the surgical procedure caused unilateral denervation of a skin zone to the left. The animals displayed paralysis and flaccidity of the abdominal muscles causing a characteristic bulging of the left side of the abdomen.

Two weeks postoperatively circular skin wounds 5 mm in diameter were cut with sharp earmark scissors on the hypaesthetic denervated side where the abdominal bulge was most pronounced. No anaesthesia was used. The contralateral intact side was similarly wounded at the corresponding site. Thus each animal served as its own control. Immediately before the operation the area was washed and shaved. The skin inside the circles was excised with fine scissors. Neither ligatures nor sutures were used.

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results from the different experiments agree so well, however, that they are considered worthy of publication. The observations have also been confirmed in a larger unpublished study by *Stillström*. He found a statistically significant scattering effect of the same type of liver extract in a concentration of 0.05. The effect of the extract was destroyed by heating to 60° C. There was no similar effect of the bile. The serum showed a less potent (but statistically significant) effect than the liver extract.

We have not learned much about this "liver factor" from these experiments, but they have shown that considerable errors are involved in calculations of the number of cells in artificial suspensions of lymphoid cells. The adding of liver extract may be a useful method by which to obtain the correct cell number and to estimate the degree of aggregation in such suspensions.

### SUMMARY

The number of cells per ccm in suspensions of thymus and lymph gland cells increased after passage through glass beads coated with water or saline extracts of liver (experiment 1 A). The total number of cells in the suspensions also appeared to increase on passage through these "liver beads" (experiment 1 B). The addition of cell-free liver extract to thymus and lymph gland suspensions increased the number of suspended cells (experiment 2 A). The cell increase occurred immediately and, as far as could be judged, was only apparent. Experiments with pipettes of different diameters support the view that the liver factor contributes to the dissolution of cell aggregates in the suspensions (experiment 2 B).

The addition of liver extract may be a useful method by which to obtain the correct cell number in suspensions of lymphoid cells and to estimate the degree of aggregation in such suspensions.

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*Figs 1-2*

- Fig 1* Aminopeptidase activity in the peripheral zone of a healthy rat skin wound age of the lesion 24 hrs  $\times 100$
- Fig 2* Aminopeptidase activity in the peripheral zone of a denervated rat skin wound age of the lesion 24 hrs  $\times 100$

cold (+4° C) neutral buffered 10 per cent formalin. The fixed specimens were further processed in the conventional way for paraffin sectioning and staining by the *van Gieson* method.

In the fresh frozen halves aminopeptidase activity was visualized by the method of *Vachlas et al* (1957), alkaline phosphatase by the technique of *Grogg & Pearce* (1952), nonspecific esterase by the  $\alpha$  naphthyl acetate modification as described by *Pearse* (1960), and cytochrome oxidase by the method of *Burstone* (1959). The 15  $\mu$  sections cut in a cryostat, were fixed for 11 minutes in cold neutral 10 per cent formalin before the demonstration of phosphatase and esterase activities. By contrast, aminopeptidase and cytochrome oxidase were visualized without any previous fixation.

## RESULTS

Two zones could be noticed around the wounds. In the immediate vicinity of the wound edge, a central or superficial zone, 200 to 500  $\mu$  in depth, showed decreasing enzymatic activity in the connective tissue cells. In this area, signs of necrosis were histologically demonstrable in 24-hour and 96-hour wounds.

Surrounding the central area, a 100 to 300  $\mu$  deep peripheral zone exhibited an increase in the enzymatic activity, initially in local fibroblasts. Additionally, in 4-hour wounds polymorphonuclear leucocytes began to immigrate the peripheral zone, contributing thus to the increased enzymatic activity. These cells were largely superseded by mononuclear cells in 24-hour and 96-hour wounds.

An increase in the activity of all of the four enzymes studied was noticed in the peripheral zone, following four hours after the injury. This zone reached a maximal stainability in 24-hour wounds, and the enzymatic activity remained strong also in 96 hour wounds.

The difference between denervated and control wounds of each animal was first seen in the enzymatic response to injury. In 4-hour wounds the activity of aminopeptidase and esterase was much less increased in the peripheral zone of the denervated wounds than in the control ones. The difference became still clearer in 24-hour wounds (Figs 1 and 2), and later on. The activation of alkaline phosphatase and cytochrome oxidase showed a similar retardation in the peripheral zones of the denervated wounds, especially from 24 to 96 hours after the injury.

There was an additional difference between the two types of wounds: also the granulocytic response to injury was distinctly retarded after denervation. In 4-hour denervated wounds immigrating polymorphonuclear leucocytes rarely occurred in the peripheral zone of the lesions (Fig. 4). By contrast, these cells were apparently more numerous in that zone of 4-hour control wounds (Fig. 3). The difference became less conspicuous in 24-hour wounds, and was hardly visible 96 hours after the injury.

## DISCUSSION

Loss of enzyme activity in the central zone apparently represents an early sign of imminent necrosis (*Rackallio* 1961, *Rackallio & Levenon*

1963 a, *Raekallio & Seligman 1964*) Later on, the necrosis could be demonstrated also histologically

The initial increase in enzyme activity of the peripheral zone seems to represent an adaptive defence mechanism (*Raekallio 1960, 1961, Raekallio & Seligman 1964*) by the local connective tissue cells, as a response to the injury. Later on, the immigrating leucocytes significantly contribute to the intensification of the enzyme activity

In denervated skin wounds, the enzymatic response to injury was retarded and less intense than normally

Considering esterases, these enzymes are known to be present in large amounts in cells engaged in histiocytic function (*Braunstein et al 1958*) The increase in esterase activity observed in the connective tissue cells in healing wounds is apparently indicative of a change in their function towards that of phagocytosis (*Raekallio & Levonen 1963 b*) In the denervated skin wounds the phagocytic ability of cells thus seems to be less pronounced than normally

Aminopeptidase activity has been related to the proliferation of the connective tissue cells (*Monis et al 1959*) In addition, this enzyme could bear a correlation to certain peptides (*Raekallio 1961*) which are known to give rise to local hyperaemia, increased capillary permeability and migration of leucocytes The last mentioned possible effect of this enzyme might be connected with the retarded granulocytic response observed in denervated wounds which simultaneously exhibit a less pronounced increase in aminopeptidase activity The weak leucocytic response, on the other hand, explains, in part, the relatively slow healing of the denervated wounds As is well known, these cells have several beneficial properties in the process of healing

Several theories exist concerning the possible functions of alkaline phosphatase *in vivo* (cf *Raekallio 1961*) *Eg Dunphy (1959)* related the activity of this enzyme in the wound to the general phenomenon of cell proliferation In fact, recent biochemical and biophysical investigations (cf *Lindner 1962*) have shown signs of proliferation in connective tissue cells, when examined 16 to 32 hours after the injury

Cytochrome oxidase participates in the respiratory processes of cells Aerobic oxidation is necessary for the occurrence of mitoses (*Bullough & Johnson 1951*) Also the activity of cytochrome oxidase, as an indicator of oxidation, was less conspicuous in the denervated wounds

Thus, by using all the four histochemical methods, the enzymatic response to injury was weaker in the denervated than in the control wounds These experiments have pointed out one possible way, enzyme histochemistry, to investigate further the complicated but interesting problem of the delayed healing of denervated wounds

#### SUMMARY

The differential enzymatic response to injury in denervated and healthy skin was experimentally investigated by using 12 albino rats





*Figs 3 & 4*

*Fig 3* Cellular reactions in the peripheral zone of a healthy 4 hour skin wound  $\times 100$

*Fig 4* Small amount of invading leucocytes in the periphery of a denervated 4 hour skin wound  $\times 100$

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## EXPERIMENTAL ABSCESES IN BRAIN AND SUBCUTIS A Microangiographic Study in the Rabbit

By

O. HASSLER and A. FORSGREN

Received 27 II 64

During the first decades of this century several experimental studies on cerebral abscesses appeared. Some of the most important investigations were those carried out by *Homen* (1912), *Groff* (1934), and *Falconer, McFarlan & Russell* (1943).

The vascular reaction plays an important rôle in abscess demarcation and healing. The purpose of this investigation is to analyse the vascular response in the neighbourhood of cerebral and subcutaneous abscesses.

### MATERIAL

Adult rabbits were used. The material was divided into a main series and an extra series. The main series comprised about fifty rabbits but several of these died of septicaemia, irrespective of the kind of bacteria during the first days after the operation. These rabbits were excluded from the study. Consequently, the main series comprised 29 surviving rabbits (see Table 1). The extra series contained 10 animals (see Bacteriological methods).

### METHODS

#### Surgical Technique

The animals were anaesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, Ill., U.S.A.). The rabbits were placed in a supine position and the lower lip was held open with a rubber band. The skin of the lower leg was shaved and disinfected with 70% alcohol. A 2 cm. incision was made in the skin of the lower leg, and the subcutaneous tissue was exposed. A subcutaneous injection of 0.05 ml. of the same agar suspension of bacteria. This injection was made deep into the subcutis of one leg.

#### Bacteriological Methods

The nutrient agar was prepared according to *Falconer, McFarlan & Russell* (1943). Thus, one bacterial colony was taken from a pure culture on an agar plate. It was inoculated in a broth tube and the bacteria were allowed to grow for 24 hours at 37° C. One drop of the bacterial suspension was inoculated into 10 ml. of a 1% agar solution. The following bacteria were used: *Proteus vulgaris*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Streptococcus pyogenes*. When the main series of rabbits was operated on, the abscesses varied somewhat and we suspected that *Falconer, McFarlan & Russell's* method of preparing the agar solution was not optimal. Therefore, the extra series was operated on with a different method of preparing the agar solution. The results of the extra series are not included in this paper.

Denervation was produced by gangliectomy on one side of the animals, the other side of each rat served as a control. Two weeks postoperatively circular skin wounds were excised on both sides. Aminopeptidase, alkaline phosphatase, esterase, and cytochrome oxidase activity was demonstrated both in the denervated and in the control wounds. The specimens were investigated also histologically. By means of all the four histochemical methods, the increase in the enzymatic activity of the peripheral wound zone was retarded and less pronounced in the denervated wounds than in the controls. In addition, a relatively scant immigration of leucocytes characterized the denervated wounds. Thus, both the enzymatic and the granulocytic response to injury was weaker in the denervated than in the healthy skin wounds.

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## EXPERIMENTAL ABSCESSSES IN BRAIN AND SUBCUTIS

### *A Microangiographic Study in the Rabbit*

By

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Received 27 II 64

During the first decades of this century several experimental studies on cerebral abscesses appeared. Some of the most important investigations were those carried out by *Homen* (1912), *Groff* (1934), and *Falconer, McFarlan & Russell* (1943).

The vascular reaction plays an important role in abscess demarcation and healing. The purpose of this investigation is to analyse the vascular response in the neighbourhood of cerebral and subcutaneous abscesses.

## MATERIAL

Adult rabbits were used. The material was divided into a main series and an extra series. The main series comprised about fifty rabbits but several of these died of septicæmia irrespective of the kind of bacteria during the first days after the operation. These rabbits were excluded from the study. Consequently, the main series comprised 29 surviving rabbits (see Table 1). The extra series contained 10 animals (see Bacteriological methods).

## METHODS

### *Surgical Technique*

The animals were short acting barb  
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100 ml of the same agar suspension of  
bacteria. This injection was made deep into the subcutis of one leg.

### *Bacteriological Methods*

The nutrient agar was prepared according to *Falconer, McFarlan & Russell* (1943). Thus, one bacterial colony was taken from a pure culture on an agar plate. It was inoculated in a broth tube and the bacteria were allowed to grow for 24 hours at 37°C. One drop of the broth culture was added to 1 ml of the nutrient agar solution. The following types of bacteria were used: *Staphylococcus aureus*, *Proteus vulgaris*, *Neisseria flavescens*, *Streptococcus pyogenes* and *Streptococcus faecalis*. When the main series had been investigated we found that the size of the abscesses varied a somewhat and we suspected that *Falconer, McFarlan & Russell's*

TABLE 1  
*Survey of the Material (Main Series)*

Rabbit No.	Type of Infection	Cereb. Injection	Sulcun- neous Injection	Time after (days)	Investigation	Cranial abscess	Sulcutaneous abscess
1	Staph aure	+	—	4	peroxid	intracereb	—
2		+	—	4			—
3		+	+	5			+
4		+	+	5	microradi		+
5		+	—	6	peroxid		—
6		+	—	8			—
7		+	—	9			—
8		+	+	10			+
9		+	+	10			+
10		+	+	11	microradi		+
11		+	—	14			—
12		+	—	15			—
13		+	+	15	peroxid		+
14		+	+	16			+
15		+	+	18	microradi	subdural	no abscess
16		+	—	19		intracereb	—
17		+	+	20		subdural	+
18		+	—	20	peroxid	intracereb	—
19		+	+	21	microradi	epidural	no abscess
20	F cells	+	+	10	peroxid	no abscess	+
21		+	+	10		intracereb	+
22	Streptoc	+	+	5			+
23		+	+	5			+
24		+	+	8			+
25	Streptoc	+	+	8			+
26		+	+	8			+
27	Proteus	+	+	10			+
28	Neisseria	+	+	10			+
29		+	+	10			+



Fig 1

A brass ring 0.9 mm high lies between the glass slide and the cover slip. The section can be removed embedded in a block of paraffin (the tissue is stained with hematoxylin and eosin).

method included a factor of some inexactness, since the number of bacteria injected was not determined, and bacteria may grow more or less rapidly in a broth owing to various factors which are not easily controlled. Therefore, 10 additional rabbits were inoculated with a known number of bacteria. Two animals received 50 milliard, two 5 milliard, two 500 million and two others 50 million *Streptococci faecalis* by each injection of 0.05 ml of the nutrient agar solution. Two other animals received sterile agar with standard broth.

Specimens were taken for culture from 7 abscesses (two subcutaneous and two cerebral abscesses caused by *Staphylococcus aureus* and further, one subcutaneous and two cerebral abscesses caused by *Neisseria flavescens*). The bacteria were identified by bacteriological techniques and were found to be the same as those injected.

### Microangiographic Methods

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lower, and from skin, muscles jaws ears and eyes The cranial cavity was opened frontally, so that the olfactory bulbs were exposed The cranium with the

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same way as previously described for peroxidase staining. After fixation the specimens were cut in a freeze microtome into slices 400-2000  $\mu$  thick. The X ray examination was performed with the aid of a Philips diffraction tube (25633/6<sup>o</sup>) with an extremely fine focus (0.16 mm<sup>2</sup>). The radiation was generated at 25 kV and 30 mA and allowed to pass through a Ni filter. The film focus distance was 100 cm. The slices were stored in thin walled plastic bags during the X ray investigation in order to prevent drying. Gevaert Scientia 5F56 or Kodak Maximum Resolution plates were used. In order to obtain a stereoscopic picture of the vascular tree the examination of the microradiograms was carried out according to Bellman (1953).

### Histological Methods

A specimen from each of the abscesses microradiographically examined and from one of the abscesses examined with the peroxidase method were embedded in paraffin cut in 10  $\mu$  thick sections and stained with the methods of van Cieson or Nissl (Romeis (1948) modification for formalin-fixed material).

## RESULTS

### Main Series

Intracerebral abscesses were obtained in all but one of the 29 rabbits. Most of the intracerebral abscesses were round or ovoid, their maximum diameters varied between 2 and 9 mm.

The vascular reaction around the cerebral abscesses was very weak. After 4-8 days a zone less than 1 mm wide of solitary dilated vessels was observed around the abscess in the peroxidase stained sections. In addition there was a diffuse oedema around the abscess (Fig. 2).

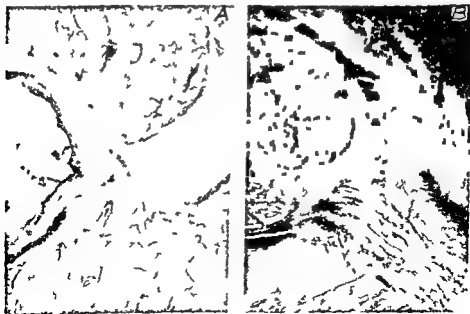


Fig. 2

Peroxidase stained frozen section through abscess 8 days after intracerebral abscess. Note the slight hyperaemia and the pronounced oedema around the abscess (to the left)  $\times 15$ . B Sulcus from the same rabbit. A distinct zone of dilated vessels (stained black) is seen at the margin of the abscess  $\times 15$ .

A



Fig 3

Microangiograms of slices 1 mm thick A Cerebral abscess, 14 days old  $\times 25$   
 B Subcutaneous abscess 14 days old  $\times 25$  C and D High magnifications of the  
 areas of newly formed vessels in A and B E and F Histological sections through  
 the same slices stained with Nissl's and van Gieson's methods respectively,  $\times 30$

After 9-11 days, there occurred in the thin capsule of the intracerebral abscesses solitary, delicate vessels, which had an anomalous course and evidently were newly-formed. After 14-16 days the zone of newly-formed vessels was less than 1 mm thick (Fig 3). However, the zone



same way as previously described for . . . . .  
 mens were cut in a freeze microto-  
 mination was performed with the  
 an extremely fine focus (0.16 mm<sup>2</sup>).  
 and allowed to pass through a Ni-f  
 slices  
 to pre  
 used  
 of the microautograms was carried out according to Bellman (1953).

n the speci-  
 X-ray ex-  
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 and 30 mA  
 100 cm. The

### Histological Methods

A specimen from each of the abscesses microradiographically examined, and from one of the abscesses examined with the peroxidase method were embedded in paraffin, cut in 10- $\mu$ -thick sections and stained with the methods of van Gieson or Nissl (Romeis (1948) modification for formalin-fixed material).

## RESULTS

### Main Series.

Intracranial abscesses were obtained in all but one of the 29 rabbits. Most of the intracerebral abscesses were round or ovoid; their maximum diameters varied between 2 and 9 mm.

The vascular reaction around the cerebral abscesses was very weak. After 4-8 days a zone, less than 1 mm wide, of solitary dilated vessels was observed around the abscess in the peroxidase-stained sections. In addition there was a diffuse oedema around the abscess (Fig. 2).



Fig. 2.

Peroxidase-stained, frozen sections through abscesses, 8 days old. A. Cerebral abscess. Note the slight hyperaemia and the spongy oedema around the abscess (to the left).  $\times 15$ . B. Subcutaneous abscess from the same rabbit. A distinct zone of dilated vessels (stained black) is seen at the margin of the abscess  $\times 15$ .

of the subcutaneous abscesses. Especially in the striated muscles which bordered on these abscesses hyperaemia was conspicuous. In the capsule of subcutaneous abscesses that were 6 days old or above several newly formed vessels were noted. These vessels were more frequent and of a coarser calibre in the subcutis than in the brain. After 18-21 days the abscesses as well as the hyperaemia and the newly formed vessels showed regression. No differences were observed between abscesses caused by various types of bacteria.

Histological sections through the abscesses showed a central core of pus surrounded by tissue heavily infiltrated with polymorphonuclear granulocytes. As is usually seen in brain abscesses there was a thin fibroblastic capsule and peripheral to it a moderate gliosis and a diffuse oedema. The capsule was a little thicker in the cortex than in the white matter. The capsule was more developed in the subcutaneous abscesses. The impressed avascular parts of the brains bordering on subdural or epidural abscesses showed a disappearance of nerve cells and gliosis (Fig. 4 B). The newly formed vessels occurred in the capsule. They had very thin walls not only in the brain but also in the subcutis. Their walls were rich in fibroblasts.

### *Results in the Extra Series*

In the two rabbits which received sterile agar no abscesses could be detected at autopsy 5 days after the injections. Of the 8 rabbits which had received *Streptococcus zymogenes* in various concentrations 3 died of septicaemia during the first days after injection. These three rabbits had received a dose of 20 milliard, 2 milliard and 500 million bacteria respectively.

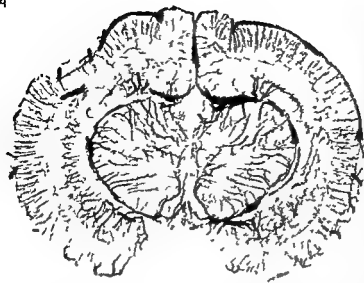
The surviving rabbit which had been given 50 milliard bacteria had very large abscesses both in the brain and in the subcutis. The cerebral abscess was rounded and about 10 mm in its largest diameter. The subcutaneous abscess was elongated (about  $20 \times 4 \times 4$  mm).

The surviving rabbit that had received 5 million bacteria had only small abscesses the size of a pin's head. The rabbit receiving 500 million bacteria had rounded abscesses which were about 5 mm in their largest diameter whereas the two rabbits which received 50 million bacteria had still smaller abscesses (about 3-4 mm in their largest diameter).

### DISCUSSION

We have not been able to find any previous report in the literature on the difference between brain and subcutis in the vascular reaction around abscesses with the exception of Greenfield's (1963). He states that the capsule is not so well developed in the brain as in other organs. Vascular reactions around abscesses have certainly not been studied with the new microangiographic methods which have been developed

A



B



Fig 4

A Microradiogram from the animal with a subdural abscess causing an impression (upwards to the left) of the brain parenchyma B A histological section through the impressed part of the same section Nissl's stain Magnification,  $\times 4$  and 30 respectively

was somewhat thicker and contained larger vessels in those parts of the margins of the abscesses, which faced the richly vascularized cerebral cortex and the large vessels at the base of the brain, than in other parts. The hyperaemia was still minimal.

After 15-18 days, the number of dilated vessels was smaller and their degree of dilatation was reduced as compared with findings after the shorter periods of observation. After 18-21 days, there was quite a definite regression of the intracerebral abscesses and of the vascular reaction around them. Three abscesses, in animals killed after 18, 20 and 21 days, respectively, had probably discharged outside the brain, so that they were situated subdurally, or in one case, even epidurally. These abscesses were encapsulated by thickened meninges. The brain parenchyma in the neighbourhood was torn and impressed (Fig 4), corresponding to the abscess. The impressed parts were poorly vascularized.

Subcutaneous abscesses were produced in all but two of the rabbits which received a subcutaneous injection of bacterial agar. These abscesses were of about the same size as the cerebral abscesses. They exerted a stronger vascular reaction than the latter. The zone of dilated vessels around the abscess was slightly thicker in the subcutaneous than in the cerebral abscesses. There was a larger number of dilated vessels, with a greater degree of dilatation, around the subcutaneous abscesses than around the cerebral abscesses. The most pronounced hyperaemia was generally observed in the deep and proximal parts of the margins

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during recent years and which afford several advantages over the classical methods (Bellman 1953, Gollman 1961, Ljungqvist 1963). In other kinds of inflammation, *e.g.* in cryptococcosis, it is also observed that the vascular reaction is much weaker in the brain than in other organs (Bergman 1961, Bergman & Hassler, to be published). We have also frequently noted that there is practically no vascular reaction around the common granulomata of *Encephalitozoonosis cuniculi* in the rabbit brain. It is not easy to determine the cause of this weak reaction of the cerebral vascular system. Within the cranium, however, space is limited and a vascular reaction, with hyperaemia and newly-formed vessels, will inevitably increase intracranial pressure and cause a compression of the remaining part of the brain. Further, the white matter develops a pronounced oedema, which is generally considered to be the most conspicuous feature on the margins of the cerebral abscesses (Wohlwill 1959). This oedema may diminish the dilatation of the vessels.

There are great differences of angioarchitecture in the cerebral cortex, the subcortical nuclei, and the white matter (Lindgren 1940, Finley 1940, van den Bergh 1961). Capillarization is much richer in the two first-mentioned locations than in the last one. In this investigation the vascular reaction to an abscess was found to be slightly more marked in the cortex than in most parts of the white matter. This is in accordance with the fact that abscesses form much more commonly in the white than in the grey matter (Greenfield 1963).

Earlier investigators (Falconer, McFarlan & Russell 1943) observed only very slight morphological differences between cerebral abscesses caused by various kinds of bacteria. Nor did we find any great differences. Probably the number of bacteria in the agar injected had some, though not a great, importance, and sterile agar caused no abscesses. Some of the types of bacteria, which we investigated, belong to those most commonly found in cerebral abscesses in man (Falconer, McFarlan & Russell, Wohlwill 1959).

#### SUMMARY

Abscesses were produced by injecting agar suspensions of various bacteria into the brains of 29 rabbits. For comparison, subcutaneous abscesses were similarly induced in most of the animals. The vascular reactions around the abscesses were studied with the aid of microangiographic methods. The hyperaemic zone was narrower and less pronounced, and the newly formed vessels were thinner and less frequent in the brain than in the subcutis. In the brain most of the newly-formed vessels were observed in such parts of the margins of the abscesses as faced the large basal arteries or the richly vascularized cerebral cortex. Abscesses caused by various bacteria showed no angiographical differences.

TABELLE

Vereinfachtes Antigen-Schema der *Salmonella* Sub-Genera II und III (1963)

O	H		O	H	
	1 Hstve	2 Hstve		1 Hstve	2 Hstve
B	H	—	11	k	Ar 25
B	e,n	l	11	L	e n
B	G	—	11	z <sub>4</sub>	—
B	G	e,n	11	H	z <sub>12</sub>
B	L	e n	11	G	l
B	z	e n	11	G	l
B	z <sub>39</sub>	l	13	G	e n
C	a	z <sub>12</sub>	13	z	l
C	a	z <sub>12</sub>	13	z <sub>4</sub>	—
C	b	e n	13	z <sub>10</sub>	l
C	b	e n z <sub>12</sub>	13	z <sub>10</sub>	e n
C	H	z <sub>12</sub>	13	z <sub>12</sub>	l
C	G	l	16	e n	l
C	G	e n	16	G	—
C	k	z	16	G	l
C	L	l	16	k	z
C	L	z	16	L	l
C	L	Ar 25	16	L	z
C	z	l	16	L	z <sub>30</sub>
C	z	z <sub>12</sub>	16	z <sub>4</sub>	—
C	z <sub>4</sub>	—	16	z <sub>10</sub>	e n
C	z <sub>30</sub>	—	16	z <sub>10</sub>	—
C	z <sub>30</sub>	z <sub>12</sub>	16	z <sub>12</sub>	l
C	z <sub>30</sub>	l	17	b	e n
C	z <sub>42</sub>	l	17	e n	l
C	z <sub>12</sub>	Ar 25	17	G	—
D	b	e n	17	G	e n
D	b	z <sub>6</sub>	17	L	—
D	c	z <sub>30</sub>	17	L	z <sub>30</sub>
D	e n	l	17	z <sub>4</sub>	—
D	G	—	17	z <sub>10</sub>	e n
D	G	e n	17	z <sub>30</sub>	—
D	G	z <sub>12</sub>	18	b	e n
D	L	e n	18	k	Ar 25
D	z	l	18	z <sub>4</sub>	—
D	z	e n	18	z <sub>10</sub>	z
D	z	z <sub>6</sub>	18	z <sub>10</sub>	z <sub>6</sub>
D	z	z <sub>30</sub>	18	z <sub>10</sub>	Ar 38
D	z <sub>4</sub>	z <sub>30</sub> z <sub>12</sub>	18	z <sub>30</sub>	—
D	z <sub>12</sub>	l	21	G	—
D	z <sub>30</sub>	l	21	z	e n
F	e n	l	21	L	z
F	G	—	21	z <sub>4</sub>	—
F	L	e n	21	z <sub>10</sub>	z <sub>6</sub>
F	L	z <sub>4</sub>	21	z <sub>10</sub>	—
F	z	l	28	b	z <sub>6</sub>
F	z	e n	28	z	z <sub>30</sub>
F	z <sub>30</sub>	z <sub>12</sub>	30	G	—
E	z <sub>30</sub>	l	35	G	—
11	d(n)	d e n			
11	G	—			
11	G	e n			
11	G	z <sub>30</sub>			

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# VIERFACHTES ANTIGEN SCHEMA DER SALMONELLA SUB GENERA II UND III

Von  
I KAUFFMANN

Eingegangen 3. 64

Im Jahre 1962 haben F. Kauffmann & R. Rohde in 2 Mitteilungen vorgeschlagen die *species* der *Salmonella sub genera* II und III (= Arizona) nur mit Hilfe des vereinfachten Kauffmann White Schemas zu diagnostizieren. Nachdem kürzlich F. Kauffmann & A. Petersen eine Tabelle mit vereinfachten *Salmonella sub genera* II Formeln (bis Ende 1962 gültig) publiziert haben sind in der folgenden Tabelle die bis Ende 1963 festgestellten *species* der *sub genera* II und III angegeben ohne aber für die Vollständigkeit der Arizona *species* zu garantieren. Es sei hervorgehoben dass hierdurch die beiden *sub genera* die nahe mit einander verwandt sind in einem einzigen Antigen Schema (dem vereinfachten K W Schema) zusammengefasst sind. Ferner sei bemerkt dass bereits mehrere dieser vereinfachten Formeln im *sub genus* I vorkommen. So entspricht zum Beispiel die Formel B<sub>12</sub> auch der vereinfachten Formel von *S. jama* und B<sub>6</sub> auch der vereinfachten Formel von *S. derby* etc. Da über die *species* des *sub genus* I im allgemeinen nach dem originalen K W Schema diagnostiziert werden spielt dieses in der Praxis keine Rolle. Um Missverständnisse zu vermeiden sollte man B<sub>6</sub> (*sub genus* II) schreiben falls es sich in diesem Falle um eine *species* des *sub genus* II handelt. In der Praxis dürfte es aber im allgemeinen genügen dem einsendenden Arzte nur die Diagnose *Salmonella sub genus* II (Enteritidis/Reger) mitzuteilen. Bis zum Beweise des Gegenteils spielt die exakte Diagnose der *sub genus* II *species* im allgemeinen keine praktische Rolle. Es ist deshalb überflüssig, derartige Kulturen nach dem originalen K W Schema zu diagnostizieren oder mit Indungen zu belegen.

O	H		O	H	
	1 Place	2 Place		1 Place	2 Place
48	k	en	55	k	z39
48	k	z39	56	l	—
48	l	z39	56	z4	—
48	L	l	57	l	en
48	L	l z47	57	z9	z19
48	r	en	57	z39	en
48	z4	—	58	l	l
48	z10	l	58	L	en
48	z10	en	58	L	z39
48	z10	—	58	r	en
48	z39	—	58	r	Ar 29
48	z39	z	58	r	Ar 29 z4
48	z39	z39	58	z9	z
50	en	l	59	k	(z)
50	G	—	59	k	z39
50	G	l	59	k	Ar 29
50	i	l	59	z4	—
50	i	z	59	z10	Ar 29
50	k	l	59	z10	Ar 40
50	k	en	59	z9	—
50	k	z	59	z39	—
50	k	z39	60	k	z
50	L	z39	60	k	Ar 29
50	r	l	60	L	z
50	r	z	60	r	en
50	z	en	60	r	z
50	z4	—	60	z	en
50	z10	—	60	z10	z
50	z10	z	60	z39	Ar 29
50	z10	z z4	61	c	l
50	z9	—	61	c	z3
50	z39	—	61	i	z
50	z39	l	61	i	Ar 29
50	z39	z	61	k	—
50	z39	z3	61	k	l
50	z3	Ar 29	61	L	l
51	G	—	61	L	z3
51	z4	—	61	r	l
51	z	z	61	r	Ar 29
51	—	l	61	z39	Ar 29
52	l	Ar 29	Ar 6	G	—
52	—	l	Ar 6	z4	—
53	G	—	Ar 8	l	—
53	i	z	Ar 8	z4	—
53	k	en	Ar 8	z39	—
53	k	z3	Ar 29	c	z
53	l	z39	Ar 29	i	z
53	r	z	Ar 29	i	z39
53	r	Ar 29	Ar 29	i	Ar 40
53	r	Ar 38	Ar 29	k	l
53	z	l			
53	z4	—			
53	z9	Ar 25			



O	H		H	H	
	1 Phase	2 Phase		1 Phase	2 Phase
35	i	—	42	I	l
35	i	e n	42	L	e n
35	i	z	42	r	—
35	k	z	42	r	Ar 25
35	k	Ar 25	42	z	l
35	L	l	42	z	e n
35	L	z <sub>30</sub>	42	z <sub>4</sub>	—
35	r	e n	42	z <sub>10</sub>	e n
35	r	z <sub>30</sub>	42	z <sub>10</sub>	z
35	r	Ar 41			
35	z <sub>4</sub>	—	43	e n	l
35	z <sub>10</sub>	z <sub>3</sub>	43	G	—
35	z <sub>9</sub>	—	43	G	z <sub>4</sub>
35	z <sub>30</sub>	l	43	I	Ar 25
35	z <sub>30</sub>	e n	43	z <sub>4</sub>	—
			43	z <sub>30</sub>	—
38	d	—	43	z <sub>4</sub>	—
38	G	—	43	z <sub>4</sub>	l
38	i	Ar 25			
38	l	z	44	G	—
38	l	z <sub>7</sub>	44	z <sub>4</sub>	—
38	k	Ar 34	44	z <sub>30</sub>	—
38	k	Ar 37			
38	L	z <sub>30</sub>	45	a	e n
38	L	Ar 25	45	a	z <sub>10</sub>
38	I	Ar 34	45	G	—
38	r	z	45	G	l
38	r	Ar 25 z <sub>17</sub>	45	G	e n
38	z <sub>4</sub>	—	45	z	z <sub>10</sub>
38	z <sub>10</sub>	z	45	z <sub>4</sub>	—
38	z <sub>10</sub>	Ar 25	45	z <sub>9</sub>	—
38	z <sub>4</sub>	Ar 25			
39	L	e n	47	a	—
			47	b	l
40	a	z <sub>3</sub>	47	c	e n
40	a	e n	47	d	z <sub>30</sub>
40	G	—	47	i	z <sub>3</sub>
40	G	e n	47	i	Ar 25
40	i	l	47	l	l
40	l	Ar 25	47	l	l z <sub>30</sub>
40	z	l	47	l	e n
40	z <sub>4</sub>	—	47	L	Ar 25
40	z <sub>30</sub>	e n	47	r	z
40	z <sub>3</sub>	—	47	r	Ar 25
40	z <sub>30</sub>	l	47	r	z
			47	z <sub>0</sub>	l
41	G	—	47	z <sub>1</sub>	l
41	k	—	47	z <sub>1</sub>	—
41	z <sub>4</sub>	—	47	z	l
41	z <sub>10</sub>	l	47	z	e n
41	z <sub>1</sub>	z			
41	z <sub>9</sub>	—	48	a	l
41	z	—	48	d	l
			48	d	z
42	b	l	48	e n	z
42	b	e n	48	G	—
42	G	—	48	i	z <sub>1</sub> Ar 40
42	k	z	48	i	Ar 25
42			48	l	l

I	II		I	II	
	1 Phase	2 Phase		1 Phase	2 Phase
48	k	en	55	III	z35
48	k	z35	56	b	—
48	k	z39	56	z4	—
48	k	Ar 25	57	z	en
48	L	l	57	z 9	z45
48	L	l z47	57	z39	en
48	r	en	58	L	l
48	z4	—	58	L	en
48	z10	l	58	L	z3
48	z10	en	58	r	en
48	z 9	—	58	r	Ar 25
48	z3r	—	58	r	Ar 25 z4
48	z42	z	58	z45	z
48	z45	z35	59	k	(z)
50	en	l	59	k	z3
50	G	—	59	k	Ar 25
50	G	l	59	z4	—
50	i	l	59	z10	Ar 25
50	i	z	59	z40	Ar 40
50	k	l	59	z 9	—
50	k	en	59	z55	—
50	k	z	60	k	z
50	k	z3	60	k	Ar 25
50	L	z35	60	L	z
50	r	l	60	r	en
50	r	z	60	r	z
50	z	en	60	z	en
50	z4	—	60	z10	z
50	z10	—	60	z 5	Ar 25
50	z10	z	61	c	l
50	z10	z z45	61	c	z3
50	z 3	—	61	i	z
50	z30	—	61	i	Ar 25
50	z55	l	61	k	—
50	z55	z	61	k	l
50	z55	z35	61	L	l
50	z55	Ar 25	61	L	z4
51	G	—	61	r	l
51	z4	—	61	r	Ar 25
51	z55	z	61	z55	Ar 25
51	—	l	Ar 6	G	—
52	L	Ar 25	Ar 6	z4	—
52	—	l	Ar 8	G	—
53	G	—	Ar 8	z4	—
53	i	z	Ar 8	z35	—
53	k	en	Ar 29	c	z
53	k	z35	Ar 29	i	z
53	l	z39	Ar 29	i	z35
53	r	z	Ar 29	i	Ar 40
53	r	Ar 25	Ar 29	k	l
53	r	Ar 38			
53	z	l			
53	z4	—			
53	z55	Ar 25			

O	H		O	H	
	1 Phase	2 Phase		1 Phase	2 Phase
Ar 29	L	z	Ar 30	k	Ar 25
Ar 29	z <sub>35</sub>	—	Ar 30	l	z
Ar 30	k	z	Ar 30	L	Ar 25
Ar 30	k	z <sub>35</sub>	Ar 30	z <sub>10</sub>	e n

## ZUSAMMENFASSUNG

Die vereinfachten Antigen-Formeln der beiden *Salmonella sub genera* II und III ( $\approx$  Arizona) werden in einer Tabelle, dem vereinfachten Kauffmann-White-Schema, angegeben

## LITERATUR

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## SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

### 6. Examination of a Selection of Previously Untypable *H. influenzae* M- and S-Strains for Evidence of Type Specific Antigens Different from the Used Type Reference Standards

By

TOR OMLAND

Received 20.1.64

In several earlier studies (Omland 1963 a, b, d) a material of *H. influenzae* strains has been examined for the occurrence of the known six type specific antigens. Out of 163 strains about one third were shown to possess such antigens, a proportion which is in good agreement with other investigations. Thus in the majority of strains no type specific antigens have been demonstrated. According to the purpose and plan of the present series of studies (Omland 1963 a) it seemed logical as a next step to search for possible antigens of similar nature, but different from the known six type substances.

## MATERIAL AND METHODS

From the original material a selection was made of strains which on morphological grounds, seemed to offer the best possibilities in a search for such antigens. The categories from which to select strains for study are illustrated in Table 1.

TABLE 1  
*Distribution of H. influenzae Strains According to Category of Variation and Serological Type as Determined by Gel Precipitation Tests*

	Serological type						Total number typable	Number untypable	Totals
	a	b	c	d	e	f			
Mucoid	5	36	0	4	2	5	52	8	60
Smooth						II	2	100	102
Totals	5	36	0	4	2	7	54	108	162*

\* The original material included also one *H.* strain (total 163)

O	H		O	H	
	1 Klasse	2 Klasse		1 Klasse	2 Klasse
Ar 29	L	z	Ar 30	k	Ar 25
Ar 29	z <sub>36</sub>	—	Ar 30	L	z
Ar 30	k	z	Ar 30	I	Ar 25
Ar 30	k	z <sub>3</sub>	Ar 30	z <sub>10</sub>	e n

### ZUSAMMENFASSUNG

Die vereinfachten Antigen Formeln der beiden *Salmonella sub genera* II und III (= Arizona) werden in einer Tabelle, dem vereinfachten *Kauffmann White Schema*, angegeben

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## SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

### 6 Examination of a Selection of Previously Untypable H influenzae N- and S-Strains for Evidence of Type Specific Antigens Different from the Used Type Reference Standards

By

TOV OMLAND

Received 20.6.64

In several earlier studies (Omland 1963 a, b, d) a material of *H. influenzae* strains has been examined for the occurrence of the known six type specific antigens. Out of 163 strains about one third were shown to possess such antigens, a proportion which is in good agreement with other investigations. Thus in the majority of strains no type specific antigens have been demonstrated. According to the purpose and plan of the present series of studies (Omland 1963 a) it seemed logical as a next step to search for possible antigens of similar nature but different from the known six type substances.

## MATERIAL AND METHODS

From the original material a selection was made of strains which on morphological grounds seemed to offer the best possibilities in a search for such antigens. The categories from which to select strains for study are illustrated in Table 1.

TABLE 1  
*Distribution of H. influenzae Strains According to Category of Variation and Serological Type as Determined by Gel Precipitation Tests*

	Serological type						Total number typable	Number untypable	Totals
	a	b	c	d	e	f			
Mucoid	5	36	0	4	2	5	52	8	60
Smooth						2	2	100	102
Totals	5	36	0	4	2	7	59	108	162*

\* The original material included also one B strain (total 163)

O	H		H	H	
	1. Klasse	2. Klasse		1. Klasse	2. Klasse
Ar 29	I	z	Ar 30	k	Ar 29
Ar 29	z <sub>10</sub>	---	Ar 30	I	II
Ar 30	k	z	Ar 30	L	Ar 29
Ar 30	k	z <sub>1</sub>	Ar 30	z <sub>10</sub>	en

### ZUSAMMENFASSUNG

Die vereinfachten Antigen Formeln der beiden *Salmonella sub genera* II und III (= Arizona) werden in einer Tabelle, dem vereinfachten *Kauffmann-White Schema*, angegeben

### LITERATUR

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*Kauffmann F. & Rohde R.* Zur Vereinfachung der serologischen *Salmonella*  
Diagnose Acta path et microbiol scandinav 56 341 342 1962

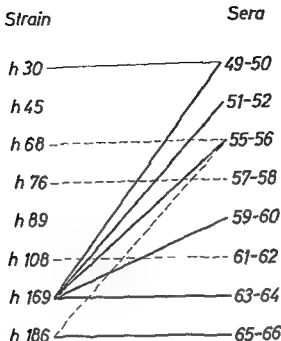


Fig 1

Homologous (horizontal lines) and heterologous (oblique lines) reactions between strains selected for examination and corresponding antisera

Broad line strong reaction  
Narrow line moderate reaction  
Dotted line weak reaction

series of gel precipitation tests was carried out in order to detect eventual subdivisions within that type. At first the whole material of 163 H influenzae strains (Omland 1963 a) was set up against the set of sera provided by dr Leidy. In addition to the e-strains already detected, the strains h 129, h 131, and h 145 (see Table 2) were found to be type e strains. An extra control was obtained by testing the sera produced by means of the strains under examination (see Fig 1) against type reference antigens (Omland 1963 d), including a reference antigen prepared in the described manner from strain h 186 and from a type e strain provided by dr Leidy (designed e Mo). These investigations verified that strain h 186 belonged to type e.

It thus appeared that two modifications of type e had been demonstrated, represented by the strains e 51 and h 186. Based upon these findings a comparison was set up of the two e-systems in gel precipitation. The result is shown in Fig 2.

According to Fig 2 it may be assumed that the type specific e antigens as prepared by the modified MacPherson's procedure contain 3 factors (indicated by roman numerals), of which h 186 possesses main-



The 8 mucoid strains untypable by gel precipitation deserved obviously a closer investigation. In addition 9 smooth strains with especially shiny colonial appearance were chosen for study. In Table 2 a review is presented of the strains subjected to examination. Additional studies were performed on strains related to type e and f.

TABLE 2  
II. *Influenzae Strains Previously Untypable by Gel Precipitation Selected for Further Examination*

	Capsular swelling untypable				Num- ber	Capsular swelling typable (type e <sup>9</sup> )				Totals	
	Strain designations					Strain designations					Num- ber
Mucoid	h 30, h 45, h 108	h 186	h 195		5	h 129, h 131	h 145		3	8	
Smooth	h 4, h 6, h 10, h 16, h 17, h 68, h 76, h 89, h 169				9					9	
Totals					14					3	17

Rabbits were inoculated with live antigens prepared from each of the listed strains with the exception of h 129, h 131 and h 145, which formed a particular group because of the results of capsular swelling typings, indicating a possible connection with type m. Two animals were inoculated with each antigen, following the procedure described earlier (Omland 1963 b), however with a reduced number of inoculations (8-10). This was done in an attempt to avoid too much antibody against antigens of non bacterial origin (e.g. components of the media) (Omland 1963 c d). Smaller volumes (0.10 ml) were also injected each time so that the total amount of antigen injected in any one animal was only about 1 ml.

Gel precipitations (miniature method, Omland 1963 c) were set up with the purpose of trying out all sera against all antigens as prepared by the modified Mac Pherson's method (Omland 1963 d).

## RESULTS

The results of the gel precipitations in the various homologous and heterologous combinations are presented in a simplified manner in Fig. 1. For practical reasons each pair of sera produced by immunization with the same antigen were employed in mixture (equal parts). Only strains or sera yielding a reaction have been included in Fig. 1.

The occurrence of partly very strong heterologous reactions was a general feature. With one exception (see below), the homologous reactions were never stronger than the heterologous ones. This fact speaks in itself against the occurrence among these strains of substances of type specific nature.

The exception was strain h 186 which gave very strong precipitation against its homologous antisera. As it was established also on repeated tests, that antigen from the said strain did not yield precipitation against any of the previously used type sera, it was tried against a set of type sera kindly provided by Dr. Grace Leidy, New York. The type e serum from this set yielded a strong precipitation against h 186.

These examinations indicated heterogeneity within type e, and a

## DISCUSSION

The occurrence among the 17 strains subject to investigation in this study, of generally stronger heterologous than homologous reactions in gel precipitations against sera prepared by the same strains, were taken as an indication that antigenic substances of type specific nature were not present.

The strain h 186 represented, however, an exception in that it yielded a very strong homologous precipitation. This fact suggested the possible existence of a type specific antigen.

Further examination of the strains by a new set of type sera resulted in the somewhat unexpected finding of four type e strains in addition to those already found, viz. the three strains already related to type e by capsular swelling tests (Table 2), and finally strain h 186. The relation of h 186 to type e was confirmed by further experiments using sera prepared by immunization with the strains under examination against type reference antigens.

According to these findings there exists two variants within type e, one variant represented by strain e 51, another by strain h 186.

The fact that 1.5 volume of ethanol is sufficient for the precipitation of the type specific factors in strain h 186, while 3 volumes are needed in strain e 51 (Fig. 2), agrees with what has already been stated in previous reports (Omland 1963 d), in which the singular position of strain e 51 in this respect is pointed out. As the so called factor I is supposed to represent the main difference in the type specific make-up of the two variants, it might be speculated whether this factor is also responsible for the higher precipitability in h 186 e.g. through a co-precipitation mechanism. Further experiments would be needed to elucidate this. It is however considered outside the scope of this study.

It seems natural to correlate these results with findings reported by Williamson & Zinnemann (1954) pointing to the presence of a twin component type specific substance in type e. Attention is also drawn to the findings described by Rosenberg *Leidy, Jaffe & Zamenhof* (1961) to the effect that the type e substance contains two monosaccharide components.

Except for the fact that all the type e strains belong to the mucoid category, there has been found no correlation between the findings reported (see Fig. 1) and the mucoid smooth differentiation (Table 2). It must however be mentioned that the strain h 169 showing particularly strong heterologous reactions, has been isolated from cerebrospinal fluid in a case of meningitis. The strain was the only untypable meningitic strain in the original material all the remaining ones being type b (Omland 1963 d).

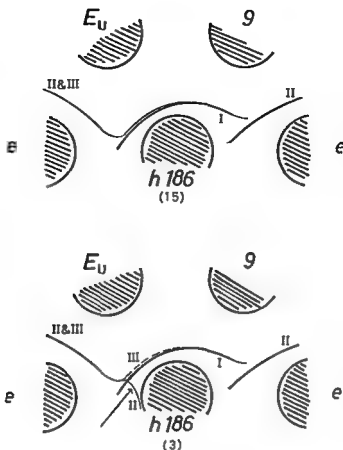


Fig 2

Differences in type specific precipitation between two type e strains  
 strain # 51 type specific reference antigen

h 186 strain h 186 type specific antigen precipitated by 1.5 vol ethanol (15)  
 and between 1.5 and 3 vol (3)

E<sub>u</sub> type e reference serum kindly provided by dr Grace Leidy, New York  
 9 own type e serum

ly I, but also considerable amounts of II, whereas e 51 possesses mainly II (traces of I). Factor III is present in both strains, but apparently in small amounts. Two fractions of the h 186 antigen have been tested, one precipitated by 1.5 volumes of ethanol (marked 15), and the other prepared by further precipitation in the supernatant up to 3 volumes (marked 3). Fig 2 shows that essentially all the factor II present in h 186 has been obtained in the 1.5 volume fraction (no identity reaction as concerns factor II between the two strains in lower figure, see arrow).

These findings of a complex type specific antigen in type e have been confirmed in experiments using ultrasonically treated suspensions of organisms as antigens (see a subsequent article, Omland 1964 a)

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## SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

### 7. Examination by Means of Gel Precipitation of Somatic Antigens Prepared by Various Disintegration Procedures in an M- and an R-Strain of H. influenzae

By

TOS OMLAND

Received 20.1.64

In the preceding studies (Omland 1963 a, b, c, d, e, 1964 a, b) the interest has been focussed on type-specific antigens and their distribution in a major material of strains.

In the following some investigations shall be reported concerning mainly the non-type-specific antigens (here called *somatic antigens*)

## MATERIAL

For examination serum no. 5 (anti H 51 serum) was employed.

## METHODS

Three disintegration techniques were used:

1. Treatment with sodium laurocholate.  
2. Ultrasonic treatment.  
3. Treatment with sodium laurocholate.

An MSE ultrasonic apparatus was used. Volumes of about 2 ml were treated each time, cooling the samples in ice water during treatment.

1. Treatment with sodium laurocholate (Pittman 1950).

The mixture was incubated

## SUMMARY

A selection of 17 *H. influenzae* strains, non-typable by previous gel precipitation tests, but growing in especially shiny colonies, has been examined for the eventual occurrence of capsular antigens different from the known type specific antigens. Antisera have been prepared to each strain, and the examinations have been performed by testing strains against sera in the various homologous and heterologous combinations. No substances of the nature mentioned have been found. However, complexities have been demonstrated in type c, and four of the strains have been shown to belong to a type c variant which was undetectable by the type c reference system used earlier.

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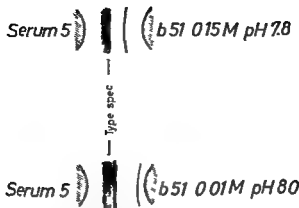


Fig 1

Gel precipitation after ultrasonic treatment for 3 minutes at pH 7.8-8.0

Effect of variation in salt concentration

Type specific precipitate partly covered by other lines (cfr. later experiments)

a definite difference between physiological and hypotonic salt concentration. The best distinction of lines was obtained at physiological salt concentration (Fig 1)

A new series of experiments was carried out to compare freshly treated suspensions with suspensions treated and then stored at  $+4^{\circ}\text{C}$  for 10 days. The centrifugation was performed at the end of the storage period. In this series the following conditions were adhered to: ultrasonic treatment for 3 minutes at a salt concentration of 0.15 M and

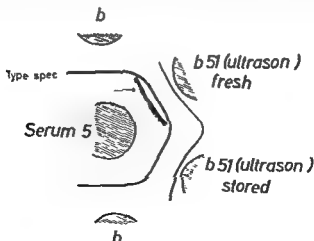


Fig 2

Gel precipitation after ultrasonic treatment for 3 minutes (0.15 M pH 7.8)

Effect of storage of antigen.

b type b reference antigen (Omeland 1963 d) Arrow indicates precipitation due to impurities from medium (horse serum antigen)

for 1 hour in a water bath at 37° C and thereafter set up in gel precipitation experiments after high speed centrifugation as described above

**Treatment with sodium carbonate** The stock suspension was prepared in the same manner as described above and a 10 per cent dilution of this suspension was prepared in 1 per cent sodium carbonate (Tunell 1953). After 1 hour on the desk the pH was lowered to 8 (from about 10) by means of 1 M acetic acid. The same centrifugation procedure was employed as described above.

**Gel precipitation** The technique described earlier (Omeland 1963 c) was used.

**Enzyme treatment** experiments to the

1:250 in a concentration of

of 0.1 ml per ml. As seen from the used concentrations a definite excess of enzyme was aimed at in order to secure the effect. The treatment was in all cases performed at 37° C for 1 hour at the standard pH described elsewhere under the respective procedures. It was thus not possible to adhere to the optimal conditions pertaining to papain (tp 52° C and pH 6-6.5).

**Microscopical examination** The suspensions were examined microscopically before and after the various disintegration procedures in order to control the effect of the treatment. Optical microscope (Gram stained smears), and in some cases also electron microscope (palladium shadowed preparations) were employed.

## RESULTS

### 1) Experiments Employing Ultrasonic Treatment

**Effect of time of treatment, salt concentration, pH, and storage of antigen suspensions after treatment (strain b 51)** Preliminary experiments had suggested as optimal a treatment period of 3 minutes as concerns the occurrence of precipitation lines. In the following experiments parallels were set up employing respectively 2, 3, and 4 minutes of treatment. The suspensions were prepared in the manner already described, and the combinations of salt concentration and pH shown in Table 1 were used.

TABLE 1

Combinations of Salt Concentration and pH in Experiments on Ultrasonic Treatment

	Total salt concentration M	
	0.15 M	0.01 M
pH	5.6	5.8
	5.9	6.1
	6.4	6.5
	6.9	7.1
	7.4	7.6
	7.8	8.0

Phosphate buffer was employed. In the series shown in the left column (0.15 M) the proportions were 0.01 M buffer + 0.14 M NaCl. In the series shown in the right column (0.01 M) the proportions were 0.005 M buffer + 0.005 M NaCl. Untreated suspensions were used as controls. The samples were set up in gel precipitations against serum no. 5 (homologous) and read after 2 days of immunodiffusion. It was found that variations of treatment within the time interval 2-4 minutes produced no appreciable changes in the results. The precipitation lines seemed also scarcely influenced by changes in pH. There was however

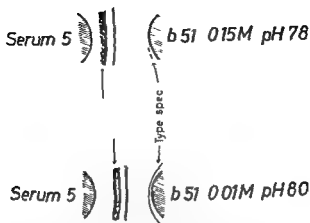


Fig 4

Gel precipitation after taurocholate treatment at pH 7.8-8.0

Effect of variation in salt concentration

Arrows indicate precipitations due partly to impurities from medium (horse serum antigens) (cfr other experiments)

In the case of bile salt treatment the trends were clear-cut, the optimal conditions being hypotonic salt concentration and high pH (0.01 M, pH 8.0). An osmotic effect is thus added to the bile salt effect. A system is seen consisting of at least two lines in the middle area. The line close to the antigen reservoir is due to type specific antigen

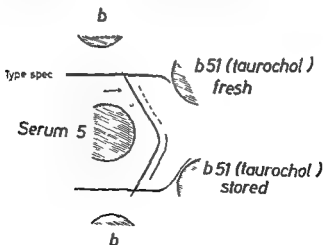


Fig 5



pH of 7.8 This pH was chosen also with a view towards later experiments using immuno-electrophoresis, which requires a high pH The results are illustrated in Fig 2

The main features of the two patterns were equal The following differences may, however, be noted the line closest to the antigen reservoir was distinctly stronger corresponding to the stored antigen A diffuse line close to the serum reservoir was on the other hand present only in the fresh antigen This line was shown to be caused by horse serum antigen (from the medium), by direct comparison in gel precipitation control experiments Figs 1 and 2 show poorer resolution of line complex near serum reservoir than indicated in later experiments

*Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R)* Based on the series of experiments described above, the following conditions for ultrasonic treatment were chosen 3 minutes' treatment at 0.15 M salt concentration and pH 7.8 Experiments were carried out in parallel using antigens prepared from each strain The results are presented in Fig 3

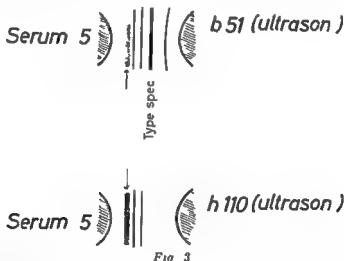


Fig 3

Gel precipitation after ultrasonic treatment (standard conditions see text)

Comparison between M strain (*b 51*) and R strain (*h 110*)

Arrows indicate precipitations due to impurities from medium (horse serum antigen)

The main elements in the two patterns were similar with exception of the type specific line The line nearest to the antigen reservoir (mentioned above) could, however, not be detected in *h 110*

The diffuse line caused by horse serum antigen was present in both strains

#### b) Experiments Employing Treatment with Sodium Taurocholate

*Effect of salt concentration, pH, and storage of antigen suspensions after treatment (strain b 51)* The suspensions were prepared as already described, and the combinations listed in Table 1 were used Fig 4 illustrates the results

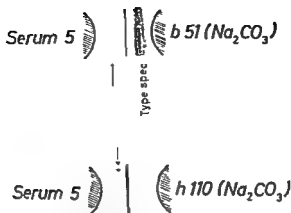


Fig 7

Gel precipitation after sodium carbonate treatment  
Comparison between V strain (b 51) and II strain (h 110)

Arrows indicate precipitations due to impurities from medium (horse serum antig)

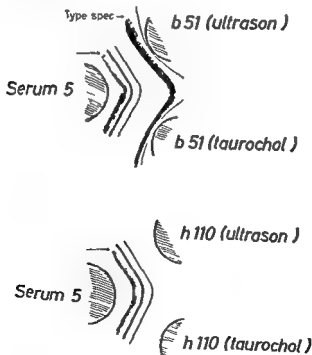


Fig 8

Gel precipitation of V and R strain

Comparison between ultrasonic treatment and taurocholate treatment of antigens  
Arrows indicate precipitations due to impurities from medium (horse serum antig)

In analogy with the storage experiments described under ultrasonic treatment some examinations were performed using freshly prepared suspensions in parallel with suspensions treated with sodium taurocholate and thereafter stored at  $+4^{\circ}\text{C}$  for 10 days. Fig. 5 shows the results.

As in the ultrasonic experiments a diffuse line closest to the serum reservoir disappeared after storage. This line was shown to be due to horse serum antigen (cf. above).

*Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R)* The optimal conditions for bile salt treatment (0.01 M, pH 8.0) were used in these examinations which were carried out in parallel in the two strains. The results are seen from Fig. 6.

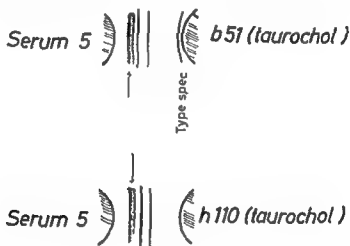


Fig. 6

Cell precipitation after taurocholate treatment (standard conditions see text).  
Comparison between M strain (b 51) and R strain (h 110).  
Arrows indicate precipitations due to impurities from medium (horse serum antigen).

As in the analogous ultrasonic experiments the main elements in the two precipitation patterns are the same excepting the type specific line which is weak even in strain b 51.

### c) Experiments Employing Treatment with Sodium Carbonate

*Patterns of somatic antigen lines in strain b 51 (M) and strain h 110 (R)* The treatment was carried out as described under Methods. The results are seen in Fig. 7.

A similar pattern to the one already recorded by the two previous disintegration methods could be seen in the middle area between the reservoirs. Also in these experiments no line close to the antigen reservoir could be detected.

treatment than after taurocholate treatment, suggesting that the former treatment yielded a more highly diffusible antigen than the latter, which might also scarcely be expected to have any direct effect on the type specific polysaccharide (cfr immuno-electrophoretic examinations, Omland 1964 b)

A diffuse, weak precipitation near the serum reservoir proved to be due to horse serum antigen. This was also in accordance with the results of enzyme treatment (see below). This precipitation tended to disappear gradually on storage of the antigen mixture (Fig 2, Fig 5). It may be assumed that autolytical processes were active during storage (cfr strengthening of the line closest to the antigen reservoir in ultrasonically treated antigens).

Treatment of the final antigen preparations by proteolytic enzymes produced no demonstrable effect on the bacterial antigen lines. DNAase was also without detectable influence. On the other hand the proteolytic enzymes affected strongly the horse serum antigen precipitation as already mentioned.

Several earlier works on non type specific *H. influenzae* antigens have been reported (see Omland 1963 a). Of the more recent studies the investigation made by Tunevall (1953) on sodium carbonate extracted antigens must be mentioned. At least two non-type specific *H. influenzae* antigens have been reported earlier (the so called M and P substances, Boyd 1956 p. 197). Dubos (1942) described the finding of an antigen supposed to be of endotoxin nature.

After the above examinations it is unlikely that the demonstrated antigens are of protein nature like the M and P substances. It seems more probable that the antigens are at least partly of polysaccharide nature.

Keeping Dubos' finding in mind, a relation to the endotoxins might be suspected. Further studies performed by aid of immuno-electrophoresis are to be published shortly.

## SUMMARY

An M strain (type b) and an R-strain of *H. influenzae* have been examined in gel precipitation experiments against an anti b serum after disintegration by ultrasonic oscillations, by bile salt, and after treatment by 1 -

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Because the

by protocol

of protein

such as the M and P substances reported by earlier authors. It is thought that they may at least in part be of polysaccharide nature (related to endotoxins?).

*Comparison of the Somatic Antigen Line Patterns as Revealed by the three Methods*

Direct comparison has been made between the patterns produced by ultrasonic and taurocholate treatment in gel precipitation experiments illustrated in Fig 8. The narrow line closest to the antigen reservoir, previously seen only after ultrasonic treatment, has been demonstrated for the first time also after taurocholate treatment.

Based on these experiments a general pattern of somatic antigen lines may be postulated, and is hereby presented schematically (Fig 9).

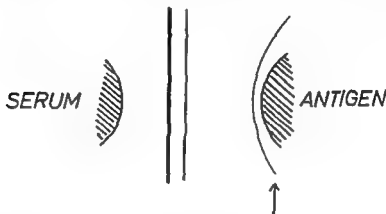


Fig 9

Schematic pattern of non-type specific (somatic) lines as revealed by gel precipitation experiments in two *H. influenzae* strains (M and R). Arrow indicates precipitation produced only by M strain.

**Enzyme treatment** The treatment by trypsin, papain, and DNase proved without demonstrable effect on the different line systems with one exception, viz the described horse serum line nearest to the serum reservoir. This line was weakened or completely removed in experiments using trypsin and papain.

## DISCUSSION

Examinations have been performed with the purpose of studying the non-type-specific antigens in a mucoid and a rough strain of *H. influenzae*. Three different disintegration methods have been employed: ultrasonic treatment, treatment with sodium taurocholate, and treatment with sodium carbonate. In addition, the effect of storage of antigen has been examined.

Ultrasonic and taurocholate treatment yielded essentially similar results, being directly comparable in gel precipitation experiments (Fig 8). The pattern caused by somatic antigens consisted thus mainly of 2 sharp lines—in the mucoid strain 3 lines—while the precipitation after sodium carbonate treatment consisted of only one line. The type specific line present in preparations from the mucoid strain was stronger and situated farther from the antigen reservoir after ultrasonic

treatment than after taurocholate treatment, suggesting that the former treatment yielded a more highly diffusible antigen than the latter, which might also scarcely be expected to have any direct effect on the type specific polysaccharide (cf. immuno-electrophoretic examinations, Omland 1964 b).

A diffuse, weak precipitation near the serum reservoir proved to be due to horse serum antigen. This was also in accordance with the results of enzyme treatment (see below). This precipitation tended to disappear gradually on storage of the antigen mixture (Fig 2, Fig 5). It may be assumed that autolytical processes were active during storage (cf. strengthening of the line closest to the antigen reservoir in ultrasonically treated antigens).

Treatment of the final antigen preparations by proteolytic enzymes produced no demonstrable effect on the bacterial antigen lines. DNAase was also without detectable influence. On the other hand the proteolytic enzymes affected strongly the horse serum antigen precipitation as already mentioned.

Several earlier works on non type specific *H. influenzae* antigens have been reported (see Omland 1963 a). Of the more recent studies the investigation made by Tunevall (1953) on sodium carbonate extracted antigens must be mentioned. At least two non-type specific *H. influenzae* antigens have been reported earlier (the so called M and P substances, Boyd 1956 p. 197). Dubos (1942) described the finding of an antigen supposed to be of endotoxin nature.

After the above examinations it is unlikely that the demonstrated antigens are of protein nature like the M and P substances. It seems more probable that the antigens are at least partly of polysaccharide nature.

Keeping Dubos' finding in mind, a relation to the endotoxins might be suspected. Further studies performed by aid of immuno electrophoresis are to be published shortly.

#### SUMMARY

An M strain (type b) and an R-strain of *H. influenzae* have been examined in gel precipitation experiments against an anti-b serum after disintegration by ultrasonic oscillations, by bile salt, and after treatment by 1 per cent sodium carbonate. A system of 2 precipitation lines (in the M strain 3 lines) has been established as being of somatic origin. Because the lines remained unaffected despite treatment of the antigens by proteolytic enzymes (and DNAase), it appears unlikely that they are of protein nature such as the M and P substances reported by earlier authors. It is thought that they may at least in part be of polysaccharide nature (related to endotoxins?).

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## SEROLOGICAL STUDIES ON HAEMOPHILUS INFLUENZAE AND RELATED SPECIES

### 8. Examinations of Ultrasonically Prepared Haemophilus Antigens by Means of Immuno Electrophoresis

By

TOV OMLAND

Received 20.1.64

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It is considered appropriate to recapitulate some of the earlier studies on non type specific *H. influenzae* antigens. Most of these studies serve to illustrate a confusing heterogeneity. Thus Park, Williams & Cooper (1918, cit. Wilson & Miles 1955, p. 909) found only 4 identical pairs among 160 strains. Labe (1921) and Krah (1930) similarly confirmed the diversity among *H. influenzae* S-strains. Iagi (= Iizuka) (1935, 1938) had to establish 46 antigenic groups in a material of about two hundred strains. Platt (1937), in a material of 87 strains, likewise failed to attain an adequate serological classification. Two non-type-specific antigens of protein nature, the so-called P and M substances have been reported (Boyd 1936, p. 197). Dubos (1942) described an antigenic substance supposedly of endotoxin character in *H. influenzae*.

#### MATERIAL

**Strains.** The strains examined have been selected from the original material (Omland 1963 a). Both typable and untypable strains have been included. A review of the strains examined is presented in Table 1.

**Sera.** The sera have been selected from a major collection because of satisfactory properties in gel precipitation and immuno electrophoresis experiments. The sera are listed in Table 2.



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Concerning the background of these investigations the reader is referred to the first report of the series (Omland 1963 a). The purpose of the immuno electrophoretic examinations was besides to inquire further into the serology of the type specific antigens of *H. influenzae* to study the non type specific antigens of that species and finally to search for related antigens in the mentioned other species of Haemophilus.

It is considered appropriate to recapitulate some of the earlier studies on non type specific *H. influenzae* antigens. Most of these studies serve to illustrate a confusing heterogeneity. Thus *Paul Williams & Cooper* (1918) and *Wilson & Miles* (1955 p. 909) found only 4 identical pairs among 160 strains. *Labe* (1921) and *Krah* (1930) similarly confirmed the diversity among *H. influenzae* S strains. *Yagi* (— *Ituka*) (1930, 1939) had to establish 46 antigenic groups in a material of about two hundred strains. *Platt* (1937) in a material of 87 strains likewise failed to attain an adequate serological classification. Two non type specific antigens of protein nature, the so called P and M substances have been reported (*Boyd* 1956 p. 197). *Dubos* (1942) described an antigenic substance supposedly of endotoxin character in *H. influenzae*.

#### MATERIAL

**Strains.** The strains examined have been selected from the original material (Omland 1963 a). Both typable and untypable strains have been included. A review of the strains examined is presented in Table 1.

**Sera.** The sera have been selected from a major collection because of satisfactory properties in gel precipitation and immuno electrophoresis experiments. The sera are listed in Table 2.

TABLE 1  
*Strains Examined by Immuno Electrophoresis*

	Number of strains
H influenzae type strains type a	2
(incl reference strains) , b	33
, c	1
, d	3
, e	1
, f	7
H influenzae non typable strains	21
H haemolyticus	1
H parainfluenzae	1
H parahemolyticus	16
Total	91

**TABL 2**  
*Sera Employed in Immuno Electrophoretic Examinations*

		Number of sera	Designations of sera								
Anti H influenzae type specific sera	a	1	3								
	b	1	5								
	c	1	15								
	d	1	16								
	e	5	9	19	23	65	66				
	f	7	10x	28	21	31	33	34	47		
Anti H influenza non type specific sera		6	35	36	38	39	42	51			
Total		22									

Designation with stable serum used in the examination of all strains listed in Table 1

## METHODS

**Disintegration** Preliminary experiments have been performed using several disintegration procedures but the present report has been based on the use of one single procedure viz ultrasonic treatment. It seemed justified to assume that conditions giving the best results in gel precipitation experiments would also be applicable in immuno electrophoretic studies (Omeland 1964b).

The organisms were seeded from freeze-dried state and grown for 18-20 hours on chocolate agar plates (heated horse blood agar without the addition of ascitic fluid) in a humid atmosphere and thereafter harvested into a minimum of fluid (physiological saline). A 20 per cent dilution of this suspension was prepared in buffered saline (0.14 M NaCl + 0.01 M phosphate buffer) at pH 8.0. The optical transmission of the suspension was registered in each experiment by aid of a Beckman mod. 6 colorimeter using a green filter (524 m $\mu$ ). The measurements were performed after a fourfold dilution of the said 20 per cent suspension (i.e. in 5 per cent of the original suspension) in order to obtain a convenient level of measurement values. After ultrasonic treatment the sample—and the untreated control—was centrifuged at high

TABLE 3  
Properties of Immuno Electrophoretic Precipitation under Different Conditions

Buffer	Final salt concentration	pH				Result
		7.0	7.5	8.0	8.6	
Acetate (HCl)	0.15 M	—	—	—	—	Fairly distinct fast migration
Acetate (HCl)	0.05 M	—	—	Fairly distinct fast migr	—	—
Phosphate (+ NaCl)	0.05 M	—	—	Fairly distinct slow migr	—	—
Phosphate	0.05 M	—	*Distinct slow migr	*Distinct slow migr	—	—
Phosphate (+ NaCl)	0.01 M	Distinct slow migr	Distinct moderate migr	Distinct fast migr	—	—
Phosphate	0.01 M	—	Distinct moderate migr	Distinct fast migr	—	—

\* Strong generation of heat --- not examined

speed (10 000–12 000 g) for 30 minutes and the supernatant subjected to immuno electrophoretic analysis

**Immuno electrophoresis** An LKB immuno electrophoresis apparatus designed for microscopical slides (26 × 76 mm) has been used. Based on the experimental conditions recommended by the manufacturer a series of experiments was carried out to adapt the technique to the special task of examining *Haemophilus* antigens. Except for minor changes the recommended basic conditions for electrophoresis and immunodiffusion were adhered to. Thus 45 minutes' electrophoresis at 250 V, and 20 hours' immunodiffusion at room temperature were employed. One per cent Difco Special Agar Noble was used for preparing the gel, which also contained Merthiolate Lilly (1:10 000) and methylvorange (3:100 000). The experiments were designed to select the optimal pH and buffer salt mixture. A homologous system consisting of strain b 51 against serum no. 5 was used as a representative test system. The recording of results was carried out by photographing the precipitation lines according to principles described earlier (Omland 1963 b). No staining was employed. The results are summarized in Table 3.

Compared to the conditions recommended by the manufacturer as good or even better distinction and separation of lines have been obtained at low salt concentrations. It was beforehand considered desirable to ensure conditions as near as possible to the conditions under which most of the previous gel precipitation studies had been carried out, so that the results might, as far as possible, be comparable. The following composition of the buffer salt mixture was then chosen: 0.01 M (0.005 M NaCl + 0.005 M phosphate buffer) and pH 8.0. The ampereage remained fairly constant during each experiment using this buffer salt mixture. It varied also only slightly from one experiment to another being in all instances within the limits 18 and 22 mA.

It was found in separate experiments that several sera contained antibodies against horse serum antigens (cf. also Omland 1964 b). The corresponding undesired precipitations could, however, be completely prevented by adding horse serum to the gel. This was done in all subsequent experiments (in a concentration of 1 per cent of the gel). At first each experiment was performed in parallel in gel with and without horse serum. Later the experiments in gel without horse serum were omitted as they furnished no extra information. The addition of human ascitic fluid was unnecessary as this was not employed as a constituent in the chocolate agar in the present series of examinations (cf. Omland 1963 c).

**Enzyme treatment** In a set of experiments the supernatants from ultrasonic treatment were exposed to the action of trypsin (Trypsin Difco 1:250) in a concentration of 0.05–0.1 mg per ml at 37°C for 1 hour before immuno electrophoretic analysis.

**Microscopical examination** of the suspensions was performed before and after ultrasonic treatment.

## RESULTS

### a) Type Specific *H. influenzae* Antigens

In all instances the type specific antigens were found not only in the ultrasonically treated suspensions, but also in the controls, i.e. in supernatants obtained after centrifugation of untreated bacterial suspensions. In this respect they differed clearly from non-type-specific antigens, which, with one exception (cf. below), were detectable in solution only after ultrasonic treatment.

As a check of the type specific properties experiments were performed cross matching all available type reference strains against the respective type sera. In each of these experiments solutions of type specific reference antigens were used as additional controls. These type reference antigens were the same as those employed earlier (Omland 1963 c).

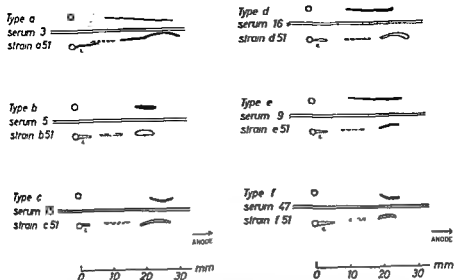


Fig 1

Immuno-electrophoretic patterns of type specific *H influenzae* antigens (schematic)  
Experiments using ultrasonically treated antigens and controls are superposed for comparison

Above serum groove type reference antigen (Omland 1963 c)

Below serum groove antigen prepared by ultrasonic treatment (continuous outline)

antigen prepared by simple dissolution (dotted outline)

Non type specific  $\epsilon$  antigen also shown

The three modifications of type specific antigens thus at disposal for examination behaved, as might be expected, in slightly different ways 1) Antigens obtained by simple dissolution (controls) These antigens demonstrated the greatest homogeneity, perhaps with the exception of the type a antigen, which precipitated in an extended line, suggesting a great variation range in electrophoretic mobility 2) Antigens obtained by ultrasonic treatment In these antigens there were, more or less in all types, signs of inhomogeneity in the form of "tail" formation towards the antigen reservoir, apparently forming a connection with the so-called  $\epsilon$  line (see below) The type a antigen showed the same tendency as mentioned above, precipitating in a line of considerable length 3) Reference antigens obtained by ethanol precipitation and phenol treatment (VacPherson 1948, Omland 1963 c) These antigens presented two different patterns, one characterized by drawn-out precipitation lines (types a, d, and e), the other characterized by precipitation spots (types b, c, and f)

The general acidic properties of the type specific antigens (Zamenhof, Leidy Fitzgerald, Alexander & Chargaff 1953, Zamenhof & Leidy 1954, Rosenberg, Leidy, Jaffe & Zamenhof 1961) were clearly demonstrated as they migrated rapidly towards the anode

Representative experiments in this series are shown in Fig 1

In view of the particular characteristics revealed by the types  $\equiv$  and  $f$  (Omland 1964 a) it was decided that immuno-electrophoretic examinations should be carried out in all available type  $e$  and type  $f$  strains against the respective type sera. The results of these examinations are reviewed in Table 4

TABLE 4  
*Type Specific Precipitation in Immuno Electrophoresis Type e and f*

Type e strains	Type e antisera					
	9	19	23	65	66	
$\equiv$ 51	+	+	+	trace	trace	
e Mo	trace	trace	trace	+	+	
h 48	trace	trace	trace	+	+	
h 131	trace	trace	trace	+	+	
h 145	trace	trace	trace	+	+	
h 186	trace	trace	trace	+	+	

Type f strains	Type f antisera						
	10x	18	21	31	33	34	47
f 51	+	trace*	+	trace*	trace*	+	+
f D1	trace	trace	+	trace	trace	+	+
h 43	+	trace	+	trace	trace	+	+
h 128	+	trace	+	trace	trace	+	+
h 147	+	trace	+	trace	trace	+	+
h 178	+	trace	+	trace	trace	+	+

\* indicates reaction between serum and strain used for immunization

It is seen that the type  $e$  strains are divided into two varieties, one represented by the type reference strain  $e$  51, yielding strong type specific precipitations against sera no 9, 19, and 23, but not against sera no 65 and 66, the other represented by the rest of the examined strains, yielding strong type specific precipitations against sera no 65 and 66, but not against sera no 9, 19, and 23

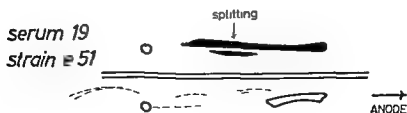


Fig 2

Immuno electrophoretic pattern showing splitting of type specific precipitation type  $e$

Above serum groove type  $\equiv$  reference antigen (Omland 1963 c)

Below serum groove antigen prepared by ultrasonic treatment (continuous outline type specific precipitation Dotted lines non type specific precipitation)

As indicated in gel precipitation experiments (Omland 1964 a), type f possesses a multiple component type specific antigen. This feature is also reflected in immuno electrophoresis showing a splitting of the type specific precipitate (Fig 2)

Within type f it has not been possible to demonstrate more than one serological variety (cfr however the f D1 serum no 10x reaction Table 4). Within this type there are however also indications of a complex type specific antigen (Omland 1963 c Fig 6) a finding which was confirmed in immuno electrophoresis. The system strain f D1 against serum no 21 is illustrative in this respect (Fig 3)

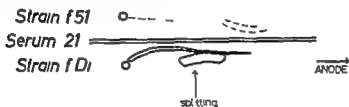


Fig 3

Immuno electrophoretic pattern showing splitting of type specific precipitation type f

Above serum groove type f reference antigen (Omland 1963 c) (homologous system)  
Below serum groove antigen prepared from another f strain (f D1) by simple dissolution (heterologous system)

## b) Non Type Specific H influenzae Antigens

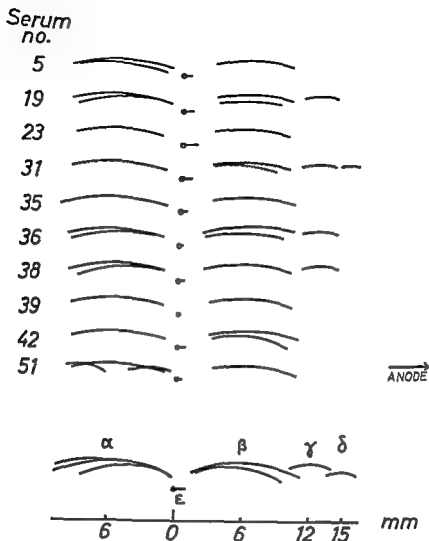
Seventy three strains have been examined by aid of the 10 selected sera

In order to create a basis for the final analysis of the antigenic pattern of each strain or group of strains the different precipitation patterns produced by the single sera were examined in an attempt to establish the characteristics of each serum. This was done by measuring the approximate distance of each line (or line complex) from the antigen reservoir. With few exceptions the lines were arc shaped and the distances could be measured from the point on the arc nearest to the serum groove and parallel to this. Minor differences in electrical strength of field and in the scale of optical recording had to be taken into consideration. Equally important was however the peculiarity of outline pertaining to each precipitation line (or line complex). These purely qualitative properties were in most instances so characteristic that it was easy to establish a precipitation pattern for the single sera representing the "total" make up of precipitins active in each

From the patterns of the different sera it was possible to fit together a hypothetical total pattern to be used as an aid in the final analysis of strains. The line patterns belonging to each serum and the hypothetical total pattern are seen in Fig 4

It is seen that all sera yielded a strong complex of lines on the





### *Synthesis of total pattern (schematic).*

Fig 4

Schematic immuno electrophoretic patterns of non type-specific (somatic) precipitations characteristic of various sera. Synthesis into theoretical total pattern

cathode side of the antigen reservoir, provisionally called the  $\alpha$  complex. The complexes situated on the anode side showed greater variations from one serum to another. Thus serum no. 31 presented a multitude of line complexes, provisionally called the  $\beta$ ,  $\gamma$ , and  $\delta$  complexes, in addition to the  $\epsilon$  line, originating in the antigen reservoir itself. Serum no. 35 is an example of a serum yielding few precipitation lines, mainly the  $\alpha$  and  $\beta$  line complexes.

The analysis of the non type-specific antigens in each strain was performed in the same way as described above concerning the fitting-

together of the hypothetical total precipitation pattern. By superposing the patterns produced in a single strain by the different sera, a picture of the antigenic equipment of that strain was brought forward.

The presentation of this rather extensive collection of single results must necessarily be problematic. In the following illustration (Fig. 5) the trends revealed by the present investigations have been placed in relation to the grouping used above (Table 1). The results have been expressed mainly in two ways, firstly in the form of numbers of antigens (as demonstrated by numbers of lines) within the different groups of strains, secondly in the form of schematic line patterns representative of each group.

In type a a comparatively simple pattern of lines was found. A most interesting feature was the demonstration of a highly soluble, non-type-specific antigenic component (precipitation indicated by arrow), possessing an electrophoretic mobility similar to the  $\delta$  complex, but otherwise different from this complex. It was thus only produced by one of the listed sera, serum no. 5 which was practically devoid of precipitation corresponding to the  $\delta$ -complex (Fig. 4). The solubility, a so far unique property among non-type-specific antigens, was demonstrated in the untreated controls, which yielded a similar line as sole precipitation, however, located somewhat nearer to the antigen reservoir.

In type b the pattern included mostly a double-line  $\alpha$  complex, a double-line  $\beta$  complex, and a single-line  $\gamma$  precipitation. In 3 strains a  $\delta$  line was clearly present. The suspensions of these 3 strains were of medium (not maximal) optical density.

In the single type c strain available a pattern similar to the type b pattern was found, except for the  $\alpha$  complex, which consisted of a single line.

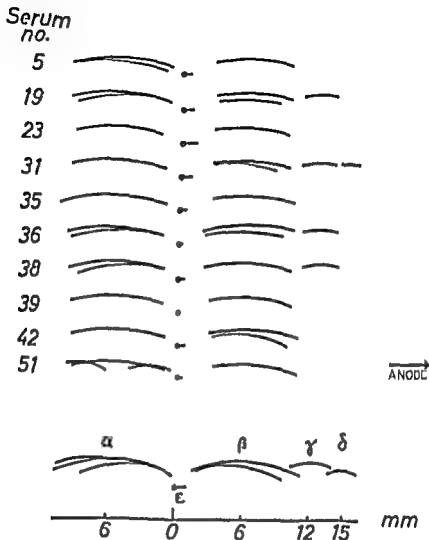
The type d pattern was similar to the type a pattern.

Type e was particularly well equipped showing double-line complexes both in the  $\alpha$ ,  $\beta$  and  $\gamma$  location, and in addition a clear  $\delta$  line in one strain.

In type f a difference was noted between strain f 51 and the other strains. While the other f strains presented a complete pattern of lines similar to the general type e pattern, the f 51 strain apparently possessed fewer antigens, mainly located in the  $\alpha$  and  $\beta$  complexes. Because of poor growth this strain gave, however, a thin suspension, so that the result may at least in part be explained technically.

The group of non-typable strains revealed a pattern which did not differ greatly from the general pattern shown by type strains. Mention should nevertheless be made of the remarkable complexity of the  $\alpha$  complex. The  $\beta$  and  $\gamma$  complexes were generally more poorly developed, while the  $\delta$  complex was not present (or only as trace in 2 strains). The  $\epsilon$  line could be clearly made out in all the above mentioned strains.

The only R strain (*Omeland* 1961a) was also examined. All the line complexes were distinct except the  $\epsilon$  line, which could be traced in



### *Synthesis of total pattern (schematic).*

Fig. 4

Schematic immunoelectrophoretic patterns of non-type specific (nontatic) precipitations characteristic of various sera. Synthesis into theoretical total pattern.

cathode side of the antigen reservoir, provisionally called the  $\alpha$  complex. The complexes situated on the anode side showed greater variations from one serum to another. Thus serum no. 31 presented a multitude of line complexes, provisionally called the  $\beta$ ,  $\gamma$ , and  $\delta$  complexes, in addition to the  $\epsilon$  line, originating in the antigen reservoir itself. Serum no. 35 is an example of a serum yielding few precipitation lines, mainly the  $\alpha$  and  $\beta$  line complexes.

The analysis of the non-type specific antigens in each strain was performed in the same way as described above concerning the fitting-

together of the hypothetical total precipitation pattern. By superposing the patterns produced in a single strain by the different sera, a picture of the antigenic equipment of that strain was brought forward.

The presentation of this rather extensive collection of single results must necessarily be problematic. In the following illustration (Fig. 5) the trends revealed by the present investigations have been placed in relation to the grouping used above (Table 1). The results have been expressed mainly in two ways, firstly in the form of numbers of antigens (as demonstrated by numbers of lines) within the different groups of strains, secondly in the form of schematic line patterns representative of each group.

In type a a comparatively simple pattern of lines was found. A most interesting feature was the demonstration of a highly soluble, non-type-specific antigenic component (precipitation indicated by arrow), possessing an electrophoretic mobility similar to the  $\delta$  complex, but otherwise different from this complex. It was thus only produced by one of the listed sera, serum no. 5, which was practically devoid of precipitin corresponding to the  $\delta$  complex (Fig. 4). The solubility, a so far unique property among non-type specific antigens, was demonstrated in the untreated controls which yielded a similar line as sole precipitation however located somewhat nearer to the antigen reservoir.

In type b the pattern included mostly a double line  $\alpha$  complex, a double line  $\beta$  complex and a single line  $\gamma$  precipitation. In 3 strains a  $\delta$  line was clearly present. The suspensions of these 3 strains were of medium (not maximal) optical density.

In the single type c strain available a pattern similar to the type b pattern was found except for the  $\alpha$  complex, which consisted of a single line.

The type d pattern was similar to the type c pattern.

Type e was particularly well equipped, showing double line complexes both in the  $\alpha$ ,  $\beta$  and  $\gamma$  location, and in addition a clear  $\delta$  line in one strain.

In type f a difference was noted between strain f 51 and the other strains. While the other f strains presented a complete pattern of lines similar to the general type e pattern, the f 51 strain apparently possessed fewer antigens, mainly located in the  $\alpha$  and  $\beta$  complexes. Because of poor growth this strain gave, however, a thin suspension, so that the result may at least in part be explained technically.

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The only B strain (Opiland 1963 1) was also examined. All the line complexes were distinct except the  $\epsilon$  line, which could be traced in

the precipitation by one serum only. All complexes were represented by single lines, with the exception of the  $\alpha$  complex, which had two lines.

A certain variation from strain to strain within each group was registered, as can be read out of Fig 5 (variation limits of numbers of lines) and Table 5. While the  $\alpha$  and  $\beta$  complexes were constantly present in all strains, inconstancy was noted in the  $\gamma$  and  $\delta$  complexes. The  $\epsilon$  line was present in all strains, although only as a trace of precipitation in the R-strain.

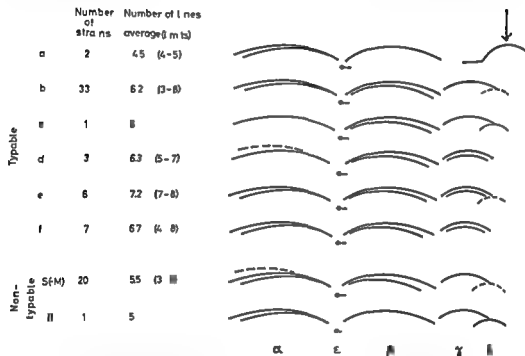


Fig 5

Schematic immunoelectrophoretic patterns of non-type specific precipitations representative of various groups of *H. influenzae* strains. Non-type specific soluble antigen indicated by arrow (see text).

TABLE 5

Subgrouping of *H. influenzae* Strains according to Non-Type Specific Pattern in Immuno Electrophoresis

Type	Number of strains	Number of different patterns
a	2	2
b	33	11
c	1	
d	3	3
e	6	3
f	7	6
Untypable	20	8

TABLE 6

*Optical Transmission Values of Suspensions of Organisms before Ultrasonic Treatment (Beckman Colorimeter Mod C)*

H influenzae type	Number of strains	Transmission values (%)	
		average	limits
H influenzae type a	2	36	33-39
b	33	27	18-36
c	1	34	
d	3	30	26-34
e	6	27	24-32
f	7	29	21-39
H influenzae untypable	20	32	20-45
H haemolyticus	1	49	
H parainfluenzae	1	86	
H parahaemolyticus	16	46	33-70

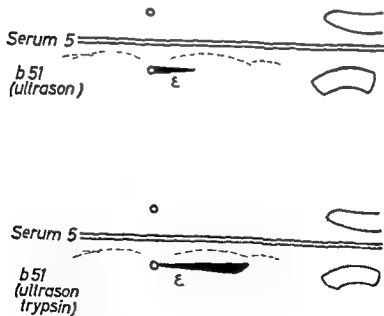


Fig 6

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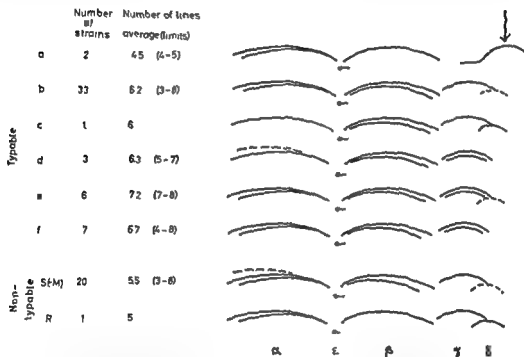


Fig 5

Schematic immuno-electrophoretic patterns of non type-specific precipitations representative of various groups of *H. influenzae* strains  
Non-type specific soluble antigen indicated by arrow (see text)

TABLE 5

Subgrouping of *H. influenzae* Strains according to Non Type-Specific Pattern in Immuno Electrophoresis

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" d	3	30	26-34
" e	6	27	24-32
" f	7	29	21-39
H influenzae, untypable	20	32	20-45
H haemolyticus	1	49	
H parainfluenzae	1	86	
H parahaemolyticus	16	46	33-70

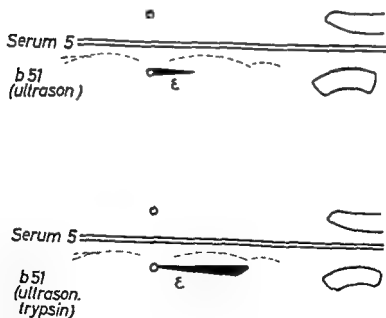


Fig 6

Effect of trypsin treatment of ultrasonically prepared antigen in a homologous  
 Groove, type II



structure?) as indicated by the location of the precipitates near the electrophoretic axis

The complexity of the type e antigen demonstrated elsewhere, has been confirmed by immuno-electrophoretic technique. By this technique it has also been possible to divide the type e strains into two variants. Previous gel precipitation analysis of representatives of these two variants (Omland 1964 a) suggests that the variant occurring most frequently in this material (represented by strain h 186) is equipped with a more complete type specific antigen (considerable amounts of two factors) than the other variant (strain e 51, mainly one factor). The fact that the strains belonging to the most common type e variant behaved differently when tested in gel precipitation against the original type reference system, strain e 51/serum no. 11 (two strains typable, three strains untypable (Omland 1963 c), suggest, however, quantitative differences even within that variant. These findings are, on the whole, in good agreement with the findings reported by Williamson & Zinnemann (1954) indicating that the complete type e antigen consists of two factors, e 1 and e 2, while there occurs also strains possessing only e 2.

In type f the findings also indicate a complex type specific antigen. Thus complexities are apparent in phenol treated antigen (MacPherson's method (Omland 1963 c)) as well as in untreated antigen solutions (Fig. 3).

It is, however, in the field of non-type-specific H influenzae antigens that the most extensive results have been secured in the present study. They lead to the establishment of a basic system of lines or line complexes, provisionally designed  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ . With the exception of the  $\gamma$  and  $\delta$  complexes, the different components appear to be present with remarkable regularity in the strains examined. This pertains to type strains as well as non-type-specific strains, and also to the R-strain studied.

Except for the particular finding in the two type a strains (cf. below), all non-type-specific antigens detected have been found only in ultrasonically treated suspensions of organisms, and not in untreated controls. This fact, and the fact that they are generally present even in the R-strain, indicates the true somatic character of these antigens, i.e. that they are firmly bound to, and eventually deeply situated in, the bacterial body, in contrast to the type specific antigens which are easily obtained by simple dissolution in accordance with their capsular character.

An outstanding feature in the serology of non-typable H influenzae strains has been the repeated finding of an extreme heterogeneity (cf. references cited in the introduction). It was natural to search among the present results for an explanation of these findings. The simplest explanation would be to assume that the registered strain-to-strain differences, notably seen in the  $\gamma$  and  $\delta$  complexes, are responsible for

the said heterogeneity. A more theoretical explanation is to postulate the presence of variable superficial masking substances, antigenic (cf. the  $\alpha$  antigen in *Salmonella*) or non antigenic under the experimental conditions. Naturally it is also thinkable that the differences underlying the heterogeneity reported by earlier authors are due to antigens not demonstrable by the methods used in the present work. It is thus recognized that as far as antigens at or near the bacterial surface are concerned, precipitation reactions are generally less sensitive than agglutination methods, on which the earlier results are based. It seems, however, improbable that the antigenic differences demonstrated previously should entirely escape registration by the methods employed in the present work.

As a final compromise in this reasoning one might postulate the heterogeneity as being due to the occurrence of a highly varied antigenic make up, however further complicated by differences in superficial masking substances.

The possibility of a variable masking effect by some less antigenic (or non antigenic) surface substance (or substances), gains support in the evidence of a non-type specific, soluble surface substance which has been demonstrated in the two type a strains. These strains grew in extraordinarily shiny colonies. The said substance was demonstrated by aid of a type b antiserum, so that the same substance, or a closely related one, must be present even in the type b strain used for immunization (b 51), however in amounts undetectable by direct precipitation. As a consequence it is also necessary to assume a greater immunogenicity in the type b strain.

Even if further evidence concerning a more widespread non-type specific surface substance (eventually non-antigenic) is lacking, it seems appropriate at this point to revert to one of the main questions posed in the introduction to the present series of works (Omland 1963 a), in which the possible existence of particular superficial antigen(s) was brought forward in order to explain the shiny colonial appearance of the S-strains.

The purpose of using *H. influenzae* sera in the study of other *Haemophilus* species was to search for eventual common antigens. Reservations must be made in the interpretations because of difficulties in securing abundant and homogenous growth.

The results show that several of the main antigen complexes found in *H. influenzae* are also present in *H. parahaemolyticus*. In the two other species, *H. haemolyticus* and *H. parainfluenzae*, the sparse material allows no further conclusions. In *H. parahaemolyticus* it may be stated, however, that the  $\beta$  complex, mostly in the form of a single line, is present in all strains. The  $\alpha$  complex is also present in a majority of strains, generally in the form of one line. Except for these two mentioned complexes the presence of lines is very inconstant from strain to strain. In two strains even the  $\epsilon$  line is present.

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In type  $f$  the findings also indicate a complex type specific antigen. Thus complexities are apparent in plicrol treated antigen (MacPherson's method (Omland 1963 c)) as well as in untreated antigen solutions (Fig 3).

It is, however, in the field of non-type-specific II influenzae antigens that the most extensive results have been secured in the present study. They lead to the establishment of a basic system of lines or line complexes, provisionally designed  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ . With the exception of the  $\gamma$  and  $\delta$  complexes, the different components appear to be present with remarkable regularity in the strains examined. This pertains to type strains as well as non-type-specific strains, and also to the R-strain studied.

Except for the particular finding in the two type  $a$  strains (cf below), all non-type-specific antigens detected have been found only in ultrasonically treated suspensions of organisms, and not in untreated controls. This fact, and the fact that they are generally present even in the R strain, indicates the true somatic character of these antigens, i.e. that they are firmly bound to, and eventually deeply situated in, the bacterial body, in contrast to the type specific antigens which are easily obtained by simple dissolution in accordance with their capsular character.

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These results are remarkable in view of the contrast, especially in morphological properties, between *H. parahaemolyticus* and *H. influenzae*.

## SUMMARY

Antigens prepared by ultrasonic treatment of 52 type strains and 21 non-typable strains of *H. influenzae*, and 18 strains of other *Haemophilus* species, have been studied by aid of 22 anti-*H. influenzae* sera in immuno-electrophoretic experiments.

The properties of the *H. influenzae* type specific antigens have been investigated, and complexities demonstrated previously, notably in type e, have been confirmed.

The non-type-specific antigens have been studied as visualized by a pattern of precipitation lines which appeared basically the same in all *H. influenzae* strains, however with a tendency to irregular variations in certain minor details. The fact that antigens from strains of other *Haemophilus* species yielded precipitations with the used sera, was taken as a proof of more widespread common antigenic factors.

In a few strains displaying particularly slimy colonies (type a) a non-type-specific, highly soluble antigen was detected. It is pointed out that the discovered substance may be a key to the explanation of certain morphological and other features peculiar to the *H. influenzae* S-form.

## GENERAL COMMENTS

The first five parts in this series of studies have been concerned with the search for type specific antigens in the material of strains and have confirmed earlier investigations showing that only M-variants possess such antigens, while the S-form, although being the most frequently occurring variant, is devoid of them. During this work particular importance has been placed in the methodological basis, because a definite negative answer to the question whether the S-variants contain type specific antigen, was decisive for the planning of further work.

In the sixth part a group of untypable strains with especially shiny colonial appearance has been examined for eventual superficial antigens other than the known type specific antigens. No such substance was detected by the used methods. However, complexities within certain serological types, notably type e, were explored.

In the seventh, and in the eighth and final part, mechanical (and other) disintegration methods have been employed in addition to the previously used chemical extraction procedures, in an effort to obtain the broadest possible display of antigens, especially of somatic nature.

Thus ultrasonic disintegration in connection with immuno-electrophoresis has been used in a number of M-strains, S-strains, and an R-strain of *H. influenzae*. A limited number of strains of other *Haemo-*

*philus* species has also been studied and antigenic relationships to *H influenzae* has been established

The results notably those attained in the present and final part justify an attempt at partial answers to the two main questions posed in the first part of the series

To the first question of whether the S-form possesses superficial antigen(s) explaining better its shiny colonial morphology, the following comments may be made Evidence has been obtained of the presence in a few M strains (type m and b) of a highly soluble superficial (?), non type-specific antigenic substance Even though this finding does not lend itself to generalization it might nevertheless be speculated if similar substances are more widespread in *H influenzae* The failure to detect such hypothetic substances may be explained by assuming either that they occur generally in very small amounts, that they are unstable or that they are not antigenic under the employed experimental conditions (cfr above on the suggested greater immunogenicity in the type b strain)

To the second question of whether the *H influenzae* variation pattern is reflected in changes in somatic antigens the following comments are offered There seems to be no direct correlation between somatic antigenic equipment and M S R variation The strain to strain differences are however, most irregular, and thus in conformity with earlier investigations

In future work a natural task would be to inquire further into the question of the postulated non type specific superficial substance(s), for instance by aid of serological adjuvant technique

Another task would be to investigate relationships to other bacterial genera such as *Pasteurella* and *Brucella*, as concerns somatic antigens

The author wishes to express his gratitude to the staff of *Kaptein W. Wilhelmsen og Frøes Bakteriologiske Institut* for cooperation advice and encouragement and in particular to the chief professor *S. D. Henriksen* for indispensable help and inspiration in outlining the project and during the work

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My thanks are hereby also conveyed to dr *Hans Chr Engbæk* *Statens Serum Institut* Copenhagen and to dr *Grace Leidy* *The Presbyterian Hospital* New York for having provided type reference strains and sera

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In a few strains displaying particularly shiny colonies (type a) a non-type-specific, highly soluble antigen was detected. It is pointed out that the discovered substance may be a key to the explanation of certain morphological and other features peculiar to the *H influenzae* S-form

### GENERAL COMMENTS

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The results, notably those attained in the present and final part, justify an attempt at partial answers to the two main questions posed in the first part of the series

To the first question of whether the S form possesses superficial antigen(s) explaining better its shrunken colonial morphology, the following comments may be made. Evidence has been obtained of the presence in a few M strains (type m and b) of a highly soluble, superficial (?), non type specific antigenic substance. Even though this finding does not lend itself to generalization, it might nevertheless be speculated if similar substances are more widespread in *H influenzae*. The failure to detect such hypothetic substances may be explained by assuming either that they occur generally in very small amounts, that they are unstable, or that they are not antigenic under the employed experimental conditions (cfr above on the suggested greater immunogenicity in the type b strain)

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## COMPARISON BETWEEN SOME STREPTOMYCIN- RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS PREPARED IN VITRO AND THEIR PARENT STRAINS

### 3 Immunological Studies

By

LENNART WAHLSTROM

Received 10 I 64

In previous papers of this serie (Norkrans & Wahlstrom 1962, Wahlstrom & Norkrans 1963) penicillin G resistant strains of *Staph aureus* and their parent strains were examined by means of the double diffusion agar technique. The results showed that the antigenic pattern of a sensitive strain underwent changes on acquiring penicillin-G resistance *in vitro*. It was also found that this was not valid for a penicillinase producer. In this paper, the study has been extended to cover also streptomycin-resistant strains, as not much has been reported about serological investigations of this type of antibiotic resistance.

The effect of streptomycin on bacteria has been examined by numerous workers. Some effects analogous to those of penicillin seem to exist *e.g.* the excretion of purine nucleotides in the presence of streptomycin and the defects it causes on the formation of the membrane (Roth *et al* 1960, Anand & Davis 1960, Feingold & Davis 1962). Hancock (1960), however, found no evidence of leakage of these compounds, using *Staph aureus* instead of *E coli*.

In their unitary hypothesis, Spotts & Stanier (1961) propose that sensitive strains have a high affinity for binding streptomycin on the ribosomes thus blocking the attachment of messenger RNA's. On the other hand, the structure of the ribosomes of the resistant strains are different having no or only a slight affinity for streptomycin. This is also in analogy with the capacity of the penicillin binding component (PBC) to bind penicillin, existing within sensitive and resistant strains, as reported by Cooper (1956). The biochemical nature of PBC is as yet unknown but is suggested to be an enzyme, located in the membrane. Recently Roman & Eriksson (1963), in studying *E coli*, have proposed

The author wishes to express thanks to Dr B Norkrans for her continued interest and advice in this work. Thanks are also due to Professor H Rylin for placing at the author's disposal accommodation for the animals and to Kahlfeld for a generous gift of streptomycin sulphate.

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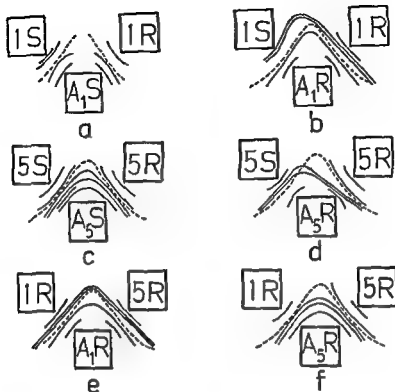


Fig 1

Precipitation patterns of the streptomycin sensitive strains 1 and 5 and their corresponding resistant strains against homologous and heterologous antisera

IS = sensitive strain 1  
 IR = resistant strain 1  
 A<sub>1</sub>S = antiserum against IS  
 A<sub>1</sub>R = antiserum against IR

5S = sensitive strain 5  
 5R = resistant strain 5  
 A<sub>5</sub>S = antiserum against 5S  
 A<sub>5</sub>R = antiserum against 5R

As seen from Fig 1, Plates a and b, the streptomycin sensitive strain 1 give five precipitation lines against both the corresponding antiserum and A<sub>1</sub>R. The reaction, however, is more vigorous in the latter case. As has been pointed out by Oeding (1960) the ability of an antigen to cause agglutination and to induce antibody formation varies from one staphylococcal strain to another. The same may be valid also for precipitinogens. In one strain the antigen may be a good precipitinogen but poorly antigenic, possibly due to the structural localization of the antigenic determinant. The knowledge in this field, however, is rather scanty.

The resistant strain 1, giving six precipitation lines against the corresponding antiserum but only four ones against A<sub>1</sub>S, shows in Plate b four shared antigenic determinants with the parent strain. The quantitative difference between two of these shared antigenic factors as revealed by the location of the two common upper precipitation lines may be due to a difference in the extractibility of these two antigens.

PBC to be a metabolite in a pathway for the synthesis of cell wall components

A comparative study between streptomycin resistant strains and their parent strains has been made by *Stern & Elek (1957)*, using agglutination reactions with absorbed antisera against heat killed bacteria. Cowan's three types were used as test organisms. They found no evidence indicating changes in the antigenic structure of the resistant variants.

This paper presents the results obtained with antisera against formalin killed bacteria tested by the double diffusion agar technique with which minute differences can be detected.

## MATERIALS AND METHODS

### Organisms

Two coagulase positive strains of *Staphylococcus aureus* were chosen for this study: strain 5 and strain 1 corresponding to Cowan's type 1. The strains have previously been examined and characterized (*Wahlstrom & Vorkrans 1963*).

### Preparation of Streptomycin Resistant Strains

Initially the resistant strains were prepared from one cell cultures of the sensitive strains by the gradient plate technique of *Szybalski (1952)* followed by repeated transfers in liquid medium of increasing streptomycin concentrations. Streptomycin was dissolved in sterile phosphate buffer pH 8.0 which was also used as the diluent. The medium consisted of Antibiotic medium 3 (Bacto Penassay Broth Difco). The strains were made resistant to 163.84 mg of streptomycin sulphate/ml.

In examining the individual antisera from the rabbits before they were pooled a variation of  $\pm 1$  in the number of precipitation lines with antisera in the corresponding tests was observed; hence a difference of one in the number of precipitation lines given by two strains cannot be considered a criterion for a real difference in the antigenic pattern as it can be caused by the variability in immune response of the different experimental animals. The following antisera were prepared: A<sub>1</sub>S = antiserum against the sensitive strain 1 (1S), A<sub>1</sub>R = antiserum against the resistant strain 1 (1R), A<sub>5</sub>S = antiserum against the sensitive strain 5 (5S) and A<sub>5</sub>R = antiserum against the resistant strain 5 (5R).

## RESULTS AND DISCUSSION

The double diffusion agar technique provides a very useful tool in separating different antigen antibody systems and has been used in numerous investigations (For references see *Ouchterlony 1962*). In comparing systems like those in this study where crude antigen solutions are used some sort of standardization is imperative. One way of doing this is to select a reference band if this is found to be caused by an antigenic determinant common to the antigen solutions tested. It should form a well recognized precipitation line with the corresponding antibody. This was found to be the case with the precipitation line in Fig. 1 marked by a dotted line. The density, time of developing and location of this reference band was found to remain constant when using an antigen dilution of 1:10 against undiluted antisera.

strains of previous studies show qualitative changes in the antigenic pattern when compared with the parent strains. Furthermore, it was not possible to find any accordance between the resistant strains, indicating different ways of acquiring this type of antibiotic resistance. A penicillinase producing strain, however, did not show any changes. The former are also supposed to develop a resistance of a more individual nature than that of penicillinase producing strains (Knox & Smith 1961).

### SUMMARY

1. The antigenic pattern of two streptomycin resistant staphylococcal strains and their corresponding parent sensitive strains have been examined by means of the double diffusion agar technique.

2. A quantitative change seems to exist in the same two antigens of the sensitive strains although no qualitative change could be found in the antigenic structure with the soluble antigenic extracts.

3. The results have been discussed in connection with earlier results obtained with penicillin resistant strains.

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from the corresponding strains. The appearance of these two precipitation lines is dissimilar—distinct between 1R-A<sub>1</sub>R, but diffuse between 1S-A<sub>1</sub>R. However, in an analogous test with strain 5, Plate d, the same situation appears, though not as pronounced as in Plate b. The lines here are quite distinct on both sides. That it is the same two antigens which cause these precipitation lines is seen in the cross-tests on Plates e and f. Here it is also seen that the quantity of these antigens is the same in the two antigenic extracts of the resistant strains. The location of the precipitation lines is different, in Plate e above and in Plate f under the "reference band", which can be explained as due to a difference in the amount of antibodies directed towards them.

Nor did strain 5S reveal any qualitative changes in the antigenic pattern on acquiring streptomycin resistance. Plate g shows seven precipitation lines between 5S-A<sub>6</sub>S and six such lines between 5R-A<sub>6</sub>S. Four of them show deviation and complete fusion, indicating at least four antigenic determinants in common. Plates c and d show the same qualitative antigenic pattern of the antigen solutions from the strains 5S and 5R against A<sub>6</sub>S and A<sub>6</sub>R, as the deviation of one precipitation line may be caused by the variation of the experimental animals (see above, p. 109).

The great similarities in the antigenic pattern between the strains is probably explained by the fact that they belong to the same phage group (Wahlstrom & Norkrans 1963).

Data presented by *e.g.* Demerec (1948), indicate that the mechanism of development of streptomycin resistance is similar to that pertaining with penicillin resistance, and that mutations are responsible for the origin of drug resistance. There is hardly any drug to which resistance emerges as readily as to streptomycin. Several genes seem to be involved in both of these two types of antibiotic resistance. In the case of penicillin resistance, all the genes have the same potency, but in streptomycin resistance they vary greatly. A high degree of resistance is therefore possible in single-step resistant strains towards streptomycin. The readiness with which the streptomycin resistant strains 1 and 5 developed, indicates them to be single-step mutants. From earlier analogous studies with penicillin resistant strains (Norkrans & Wahlstrom 1962, Wahlstrom & Norkrans 1963), there is no doubt that they acquired a high degree of resistance by a multi-step process.

In this study, limited only to a recording of the number of precipitation lines, there is no evidence of a qualitative change in the antigenic pattern of a sensitive strain on acquiring streptomycin resistance. As has been discussed, it cannot be excluded, however, that a quantitative change does in fact occur. The acquisition of streptomycin resistance, proposed to be caused by a single-step mutation, seems to be reflected in the same two common antigenic determinants of the strains tested, thus indicating a common resistance mechanism.

On the other hand multi-step mutants such as the penicillin resistant

### The indirect Proof

If two or more hypotheses could be used, and these hypotheses 1) exclude each other and 2) are all hypotheses possible, one of these hypotheses may sometimes be proved indirectly, namely if the other hypotheses can be rejected using usual significance tests. As a further consequence the hypothesis proved by this method does not need to say anything about the character or distribution of the observations, whereas this is necessary for the hypotheses to be rejected.

Significance tests of usual type ( $\chi^2$ ,  $v$  etc (see for instance Fisher & Yates (6))) are in a way indirect proofs: it is rejected, that two groups are identical and then they must be different. These tests are frequently used in biological work, but the employment of the indirect proof mentioned above is rarely met.

Two examples will be quoted.

A) In the discussion about the origin of staphylococcal infection the possibility of self infection has played an important role since Danbolt (4) reported 24 cases of cutaneous infection in patients, who carried the infective type in their nose (carriers). It has been stressed in support of this hypothesis, that infections are more frequent in carriers than in non carriers, 7 and 2 per cent respectively (Williams *et al* (10)), and that the patients were carriers before infection occurred.

These statements are certainly in accordance with the hypothesis of self infection and if they were not the hypothesis had already been rejectable. But it is not surprising, that carriers are susceptible to infection, both conditions supposedly being determined by the resistance of the patients. And the infected patients might by chance be infected from outside by the type of *Staphylococcus aureus* already resident in their nose. And this is the alternative hypothesis.

Do nasal carriers of *Staphylococcus aureus* get their wounds infected with staphylococcal strains from other persons and not from their own nose?

To try this hypothesis it would be necessary to know the carriers and clinically infected persons among other patients, nurses and doctors. In each case of infection the probability that the patient had by chance acquired the nasal type from outside could be estimated from the number of persons harbouring the incriminated type as numerator, and the total number of carriers and septic persons as denominator.

This could be done in an investigation carried out 1957-59 at Rigshospitalet in Copenhagen (Siboni (8)). 37 of the infected patients were nasal carriers before the infection occurred. Among these 37 patients 28 became infected with the type of *Staphylococcus aureus* already inhabiting their nostrils. If the presence of the nasal strain of *Staphylococcus aureus* in the wound had been determined by chance contraction from outside this would have happened in only 5.5 cases. The difference between these figures is highly significant ( $t = 5.3$ ), and self-



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## ON ARGUMENTATION IN BIOLOGY

### *The Indirect Proof*

By

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In biology including medicine as in other empiric sciences argumentation cannot be exclusively deductive. The elements of deduction are found in Euclid's geometry, which leaves no doubt that the triangles are really congruous.

In the empiric sciences a hypothesis is deduced from some premises because the investigator in a set of observations has observed a certain trend. New sets of observations are necessary to decide if the hypothesis may be correct. Disagreement between the new observations and the hypothesis results in the rejection of the hypothesis. The evaluation of this disagreement is statistical: one of the usual significance tests gives the probability to find the given set of observations if the hypothesis is true. If this probability is sufficiently small the hypothesis is rejected. The criterion for rejection is, therefore, quantitative and more or less rigorous, most often a probability of 1 or 5 per cent for deviation by chance.

If the probability is larger the hypothesis is not rejected. In this situation it is often concluded, that the hypothesis has been proved, in analogy to the congruous triangles. But this is not necessarily true, for using the premises one could most often construct several hypotheses agreeing with the observations done. Green (7) wrote: "A sufficiently ingenious mechanic could separate the parts of a baby Austin and use them to make a perambulator or a pressure pump or a hair dryer of sorts. If the mechanic was not particularly bright and was informed as to the source of these parts he might be tempted into believing, that they were in fact designed for the particular end he happened to have in view."

Fisher (5) formulates the agreement as the "null hypothesis" and writes: "it should be noted, that the null hypothesis is never proved or established but it is possibly disproved in the course of experimentation."

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Two examples will be quoted.

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infection must have occurred in approximately 22 out of the 37 cases (28 - 5.5)

B In the evaluation of blood cultured for bacteria contamination causes great difficulty as its frequency amounts to 5-15 per cent in different series, i.e. of the same size or even more frequent than growth due to bacteraemia in the patients

When *Staphylococcus aureus* (coagulase positive) is found, the reason is most often a real bacteraemia, and the patients are severely ill, but *Staphylococcus aureus* is widely distributed among carriers and has a fair chance to be a contaminant. It is, therefore, important to get a criterion for growth on account of bacteraemia

In an investigation at Blegdamshospitalet in Copenhagen (Siboni (9)) blood cultures were normally done at least two times from each patient. In 1470 blood cultures from about 500 patients *Staphylococcus aureus* was found 91 times, which gives an average frequency per blood culture of 0.062. In 15 patients *Staphylococcus aureus* was found at least two times in subsequent cultures, in 8 patients in one of two subsequent blood cultures and in 2 patients once in one blood culture.

The hypothesis alternative to bacteraemia is contamination

In order to see the distribution of a contaminant, *Staphylococcus albus* may be examined, as *Staphylococcus albus* judged from the clinical state of the patients in no case of this series was the cause of disease. *Staphylococcus albus* is here defined as a Gram positive coccus growing in clusters and coagulase negative. It was found 122 times, which gives an average frequency per blood culture of 0.083. In 10 patients *Staphylococcus albus* was found at least two times in subsequent cultures, in 66 patients in one of two subsequent blood cultures and in 25 in a single culture.

TABLE 1

*Distribution of Staphylococcus aureus and Staphylococcus albus among 1470 Blood Cultures from 500 Patients*

	2 subsequent cultures positive	1 positive of 2 subsequent cultures	1 single culture positive
<i>Staphylococcus aureus</i>	15	8	2
<i>Staphylococcus albus</i>	10	66	25

It is seen that the distribution of *Staphylococcus aureus* is entirely different from that of *Staphylococcus albus*, even if the last column of Table 1 is excluded ( $\chi^2 = 25.5$ ,  $F = 1$ ,  $P < 0.05$ ), and it may be concluded that *Staphylococcus aureus* was not distributed as the contaminant *Staphylococcus albus*. A distribution at random among the blood cultures of a bacterium found with a frequency averaging 0.083 would 3-4 times give growth in both of two subsequent cultures and 76 times

in one of two. These figures correspond closely to those of *Staphylococcus albus*.

If the 8 cases where *Staphylococcus aureus* was found in one of two subsequent cultures were to be regarded as a result of contamination, the frequency,  $p$ , of this contamination per blood culture would be determined by  $2p(1-p) \cdot 500 = 8$  hence  $p = 0.008$  and this would be the maximal frequency for *Staphylococcus aureus* as a contaminant. The probability to find *Staphylococcus aureus* in two subsequent cultures on account of contamination would be  $0.008^2 = 0.000064$  and growth would appear in 0.032 among 500 patients. Actually *Staphylococcus aureus* was found in 15 cases in at least two subsequent cultures.

Conclusion. *Staphylococcus aureus* does not occur in blood cultures as a randomly distributed contaminant, i.e. when found it is normally a cause of disease. The criterion for bacteraemia also appeared: growth of *Staphylococcus aureus* in two subsequent blood cultures means bacteraemia as this could be due to contamination only once among 15 625 pairs of blood cultures.

## DISCUSSION

In example B blood cultures the alternative hypothesis of contamination in a way expresses the error of the method. It is well known from the analysis of variance to subtract this error from the total variance in order to determine the part of the variance caused by the investigated variable itself. But the mere fact, that this variable exists and has a variance normally does not prove any hypothesis about it which is the case here. And in example A self infection or not, the alternative hypothesis does not express the error, it is only a negation of the original hypothesis.

It is a consequence of the quantitative character of significance tests that the alternative hypothesis cannot be rejected as a whole. The alternative hypothesis may be correct as regards some of the observations. But the rejection means that the alternative hypothesis does not satisfy the total set of observations and that the main hypothesis is correct at least concerning a part of the observations. The size of this part can be estimated as done in example A.

An important condition for the use of the indirect proof is certainly that the alternative hypothesis is correctly laid down. If not its rejection is of no value and the whole proof is useless. In example A it may be discussed if it is correct to use the total number of carriers and patients with septic lesions as the denominator in the estimate of the probability to get the nasal type by chance from outside as all types might not be infectious to the same degree and some of them perhaps not at all. This could be compensated and also the infections were caused by a diversity of types.

In example B it could be asked how one would know if the occurrence

infection must have occurred in approximately 22 out of the 37 cases (28 - 5.5)

II In the evaluation of blood cultured for bacteria contamination causes great difficulty as its frequency amounts to 5-15 per cent in different series, i.e. of the same size or even more frequent than growth due to bacteraemia in the patients

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## IMMUNOCHEMICAL STUDIES ON ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS

### 2 Precipitating and Erythrocyte Sensitizing Properties of Protein A (Antigen A) and Related Substances

By

PER ODFINE ARNE GROV and BERIT MYKLESTAD

Received 10. 64

Antigen A of *Staphylococcus aureus* has been isolated in a relatively pure state by us and characterized chemically (2). While Jensen (9, 10, 11) considered the antigen to be a polysaccharide we in agreement with Ickhust & Sjöquist (14) found that it is a protein which is probably identical to the protein fraction B described by Verwey in 1940 (23). We proposed to call this antigen *protein A* to avoid confusion with polysaccharide A and antigen B.

Jensen *et al.* (11) demonstrated that their extract A contained at least three different components: 1. Antigen A characterized by its precipitation line on agar with normal human sera; 2. Toxin D; 3. A heterogeneous antigen of the Rantz type.

Erythrocyte-sensitizing antigens have also been demonstrated in extracts of staphylococci by other authors (2, 21, 19, 17, 5, 15).

The intention of the present investigation has been to examine the serological properties of our purified protein A preparation. As it was shown to contain in addition to its precipitinogen an antigen that sensitized tanned erythrocytes, it was decided to examine the presence in extract A and polysaccharide A of sensitizing substances and to attempt to correlate our findings with earlier reports.

## MATERIALS AND METHODS

### Strains

The *Staph aureus* strains used belong to our typing set (8). Strain Cowan I (NCTC 8330) was used to prepare extract A and C.

of *Staphylococcus albus* in two subsequent blood cultures was caused by bacteremia. The answer is that *Staphylococcus albus* defined as here is a very heterogeneous group of the Micrococccaceae which ought to be divided using the criteria in *Bergey's Manual of Determinative Bacteriology* (2) or as *Baird Parker* did it (1). A division of this kind combined with sensitivity tests would yield numerous types of *Staphylococcus albus* making the probability to refind the same type very small.

The qualitative character of epidemiology perhaps makes it especially accessible to the use of the indirect proof but it could also be used within other parts of microbiology such as in the distinction between mutation and adaptation if the consequences of one of the two hypotheses were rejectable (*Burnet* (3)).

The indirect proof has supposedly been used within other fields of the natural sciences.

### SUMMARY

It is mentioned that the accordance of a set of observations with the hypothesis is no proof for the correctness of this hypothesis while disagreement between observations and hypothesis disproves the latter.

Under special circumstances it is nevertheless possible to prove a hypothesis indirectly namely when there are only one or a few alternative hypotheses and these can be rejected (The indirect proof).

Two examples are discussed.

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## MATERIALS AND METHODS

### Strains

The *Staph aureus* strains used belong to our typing set (8). Strain Cowan I (NCTC 830) was used to prepare extract A and protein A. Its serological pattern is as follows:

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40. 41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60. 61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80. 81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100.



of *Staphylococcus albus* in two subsequent blood cultures was caused by bacteraemia. The answer is, that *Staphylococcus albus* defined as here is a very heterogeneous group of the Micrococaceae, which ought to be divided using the criteria in *Bergey's Manual of Determinative Bacteriology* (2) or as *Baird-Parker* did it (1). A division of this kind combined with sensitivity tests would yield numerous types of "Staphylococcus albus", making the probability to refind the same type very small.

The qualitative character of epidemiology perhaps makes it especially accessible to the use of the indirect proof, but it could also be used within other parts of microbiology such as in the distinction between mutation and adaptation, if the consequences of one of the two hypotheses were rejectable (*Burnet* (3)).

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It is mentioned that the accordance of a set of observations with the hypothesis is no proof for the correctness of this hypothesis, while disagreement between observations and hypothesis disproves the latter.

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human serum (see Fig 2 in *Grov et al* (2)) The other line, appearing only in Cowan I antiserum was stronger and sharper than the first one This line could not be identified with any other known staphylococcal antigen

As the double line appeared regularly in all our Cowan I antisera and was found with extracts of other *Staph aureus* strains than Cowan I, it did not seem to be artificial However, we did not succeed in separating the two lines on ion exchange columns on electrophoresis, or on immunoelectrophoresis Both disappeared when the material was digested with trypsin

#### *Sensitization of Normal Sheep Cells*

When 0.1 ml of packed sheep cells were treated with 0.2 mg of extract A the cells were sensitized to agglutination in Cowan I antiserum but not in a pool of normal human sera or in 10 sera from blood donors The haemagglutinin titre in Cowan I antiserum was 1:280 When washings from agar cultures or crude extracts were used all the *Staph aureus* strains examined had the ability to sensitize normal sheep cells, and haemagglutinins were found in the rabbit immune sera (Table 1) The strains seemed to contain varying amounts of the antigen The two *Staph epidermidis* strains and the two *M. subtilis* strains examined, but not the one strain of *M. lysodeikticus*, also sensitized normal sheep cells to agglutination in Cowan I antiserum

However the ability to sensitize normal sheep erythrocytes was not demonstrated in the purified protein A, in polysaccharide A, in polysaccharide B or AC isolated from *Staph epidermidis* (12), or in preparations of *Staph aureus* wall teichoic acid containing both  $\alpha$ - and  $\beta$ -N-acetylglucosamine residues

TABLE 1  
*Haemagglutination of SSSC*

| Materials used for sensitization          | Haemagglutinin titres<br>(reciprocal values)<br>in Cowan I serum |
|---|--|
| <i>Staph aureus</i> Cowan I crude extract | 1280   |
| " " Cowan I extract A                     | 1280   |
| " " Cowan I extract B                     | -  |
| " " "                                     | 640  |
| " " "                                     | -  |
| " " "                                     | -  |
| " " "                                     | 640  |
| " " "                                     | 2560   |
| " " "                                     | -  |
| " " "                                     | 1280   |
| " 125% polys B                            | -  |
| <i>M. subtilis</i> 10465 washings         | 2560   |
| " 1 washings                              | 1280   |
| <i>M. lysodeikticus</i> 2665 washings     | -  |

SSSC = Sensitized normal sheep cells

### Antigenic Materials

Purified protein A was prepared according to the method used by us (2) and extract A (crude protein A) according to *Jensen's* procedure (10). Polysaccharide A was obtained using the purification procedure of *Haukenes* (3). Polysaccharide B and polysaccharide AC were prepared according to the method of *Losnegard & Oeding* (12). Washings from agar cultures and crude extracts were prepared from a number of the strains mentioned above.

Teichoic acid samples from *Staph aureus* cell walls were kindly furnished by Professor *J Baddiley* Newcastle and by Professor *J I Strominger* St Louis.

### Immune Sera

Rabbit immune sera against strains Cowan I Wood 46 and other *Staph aureus* type strains and against the four *Staph epidermidis* strains were produced by intravenous injections of formalin killed bacteria as described by *Oeding* (18). The same pool of 10 normal human sera was used in all experiments. In addition individual sera from blood donors were examined.

Immune serum against the antigen in polysaccharide A which sensitizes tanned sheep cells, was prepared in the following way. Tanned sheep erythrocytes were sensitized with polysaccharide A washed three times with saline, and made up to a 10 per cent solution in saline. Rabbits were given three series of intravenous injections each series consisting of three injections. The injections were given daily with 5 days' interval between the series. Each dose contained 1 ml of erythrocyte solution. Before use the serum was absorbed with normal sheep cells.

### Methods

The ring test precipitation was carried out according to the method used by *Haukenes et al* (7) and the agar precipitation was that used by *Haukenes & Oeding* (8).

A 1:40 000 solution of tannic acid was used for the preparation of sheep cells. The sensitization of normal and tanned sheep cells was performed essentially as described by *Morse* (15). Usually 0.1 ml of packed erythrocytes were sensitized with 0.2 mg of antigenic material. The sera were diluted in tubes to a total volume of 0.5 ml. To each tube was added 0.1 ml of a 1.25 per cent solution of the sensitized cells. After thorough mixing the tubes were placed in a water bath at 37°C for 30 minutes and then at room temperature over night. The haemagglutination was recorded before and after light shaking of the tubes.

Absorption of sensitizing antigen from the preparations was performed with large doses of normal or tanned sheep cells in a 37°C water bath for 30 minutes. The complete removal of sensitizing antigen was controlled by testing the sensitizing ability of the absorbed preparation and the haemagglutinating ability of the cells used for absorption.

Absorption of antibodies against the sensitizing antigens was performed by adding a surplus of sensitized normal or tanned sheep cells to the serum diluted 1:2 and incubating at 37°C for 30 minutes. Complete absorption was controlled by testing the sensitized cells used for absorption and the haemagglutinating ability of the absorbed serum.

## RESULTS

### Precipitation

The purification of protein A on DLAL cellulose column led to an increasing ring test titre of the material. Where is the ring test titre of extract A in a concentration of 1 mg/ml was only 1:10 with Cowan I antiserum, the titre of the purified protein A was 1:80.

On agar precipitation both protein A and extract A gave two closely situated lines with Cowan I antiserum but only a single line with normal human serum. Other lines were not observed. The lines of the two products showed reactions of identity. The line closest to the antigen well gave a reaction of identity with the single line appearing with the

showed that it contained 5 amino acids (Lys, Gly, Ser, Glu A, and Ala) and no sugar. The sensitizing ability was destroyed by trypsin (Table 4). These experiments thus confirm the results of the absorptions in that the antigen in extract A (and protein A) sensitizing tanned sheep cells is a distinct antigen. The antigen is of protein nature.

TABLE 2  
*Haemagglutination in Rabbit Immune Serum and Human Serum*

| Sensitized sheep cells | Haemagglutination titres (reciprocal values) in sera |         |                  |       |
|------------------------|--|---------|------------------|-------|
|                        | Cowan I  | Wood 46 | Polysaccharide A | Human |
| Extract A STSC         | 2560   | 80      | 40               | 1280  |
| Extract A NSC          | 1280   | 160     | —                | —     |
| Protein A STSC         | 20480  | 80      | 40               | 10240 |
| Polysaccharide A STSC  | 80   | 20480   | 80               | 640   |

STSC = Sensitized tanned sheep cells

NSC = Sensitized normal sheep cells

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells

TABLE 3  
*Absorption of Preparations with Tanned Sheep Cells*

| Preparations     |              | Precipitation in homologous serum* (reciprocal values) |      | Haemagglutination in homologous serum* (reciprocal values) |      |
|------------------|--------------|--|------|--|------|
|                  |              | Ring test  | Agar | STSC   | NSC  |
| Polysaccharide A | Not absorbed | 3000   | +    | 20480  | —    |
|                  | Absorbed     | 3000   | +    | —  | —    |
| Protein A        | Not absorbed | 80   | +    | 20480  | —    |
|                  | Absorbed     | 80   | +    | —  | —    |
| Extract A        | Not absorbed | 10   | +    | 2560   | 1280 |
|                  | Absorbed     | 10   | +    | —  | 320  |

STSC = Sensitized tanned sheep cells

NSC = Sensitized normal sheep cells

\* Polysaccharide A examined in Wood 46 serum, protein A and extract A in Cowan I serum

Our polysaccharide A preparation also sensitized tanned sheep cells (Table 2). Complete sensitization of 0.1 ml of packed sheep cells was obtained with 0.02 mg of polysaccharide A. The haemagglutinin titre in Wood 46 antiserum was 1:20480 using standard conditions and even 1:40960 when the erythrocyte concentration was decreased to 1 p.c.

Protein A is prepared

from strain Cowan I, from which pro-

The ability to sensitize normal sheep cells was destroyed at 100° C at alkaline pH but not at acid pH. It was resistant to trypsin (Table 4). These properties and the distribution of the antigen indicate that it is of the Rantz type (21).

The antigen sensitizing normal sheep cells could be separated from the precipitinogens protein A and polysaccharide A on ion exchange fractionation of crude extracts. An experiment was described in part 1 of this investigation (2), in which chromatography of extract A resulted in the separation of 3 fractions. One fraction contained only the antigen sensitizing normal sheep cells, the second fraction contained only an antigen sensitizing tanned sheep cells (see below), and the third fraction contained the precipitinogen (protein A) in addition to minor amounts of the two sensitizing substances. Chemical examination of the fraction containing only the antigen sensitizing normal sheep cells showed that it contained ribose, glucuronic acid, and only small amounts of three amino acids.

Absorption of extract A with normal sheep cells, removed all antigen sensitizing normal sheep cells while the precipitin titre (protein A) and the ability to sensitize tanned sheep cells remained unchanged. The absorption experiments and the separation on DEAE cellulose column thus show that the antigen sensitizing normal sheep cells is not identical to any other antigen present.

### *Sensitization of Tanned Sheep Cells*

Extract A and protein A sensitized tanned sheep cells to agglutination both in Cowan I antiserum and in normal human serum. When 0.1 ml of packed, tanned erythrocytes were treated with 0.2 mg of protein A (or extract A), the haemagglutinin titre of Cowan I antiserum was 1:20,480 to 1:2,560 and that of normal human serum of the same order (Table 2). When extract A was absorbed with large doses of tanned erythrocytes, all antigen sensitizing tanned cells was removed while the antigen sensitizing normal cells and the precipitinogen were still present (Table 3). Absorption of Cowan I antiserum with tanned erythrocytes sensitized with extract A, removed the corresponding antibodies, but not antibodies against normal erythrocytes sensitized with extract A or against the precipitinogen. Thus the antigen sensitizing tanned sheep cells is not identical to the antigen in extract A which sensitizes normal cells, or to the precipitinogen (protein A). The antigen sensitizing tanned cells demonstrated in extract A and in protein A were shown by absorption to be identical.

In the fractionation experiment on DEAE cellulose column reported in (2), the material sensitizing tanned cells could be separated from the two other antigens present in extract A. The separated material was only able to sensitize tanned sheep cells, and gave no precipitation on the ring test or on agar. The chemical examination of the fraction

showed that it contained 5 amino acids (Lys, Gly, Ser, Glu A, and Ala) and no sugar. The sensitizing ability was destroyed by trypsin (Table 4). These experiments thus confirm the results of the absorptions in that the antigen in extract A (and protein A) sensitizing tanned sheep cells is a distinct antigen. The antigen is of protein nature.

TABLE 2  
*Haemagglutination in Rabbit Immune Sera and Human Serum*

| Sensitized sheep cells | Haemagglutinin titres (reciprocal values) in sera |         |              |        |
|------------------------|---|---------|--------------|--------|
|                        | Cowan I   | Wood 46 | Poly A STSC* | Human  |
| Extract A STSC         | 2 560   | 80      | 40           | 1 280  |
| Extract A SASC         | 1 280   | 160     | —            | —      |
| Protein A STSC         | 20 480  | 80      | 40           | 10 240 |
| Poly A STSC            | 80  | 20 480  | 80           | 640    |

STSC = Sensitized tanned sheep cells

SASC = Sensitized normal sheep cells

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells

TABLE 3  
*Absorption of Preparations with Tanned Sheep Cells*

| Preparation |              | Precipitation in homologous serum* (reciprocal values) |      | Haemagglutination in homologous serum* (reciprocal values) |       |
|-------------|--------------|--|------|--|-------|
|             |              | Ring test  | Agar | STSC   | SASC  |
| Poly A      | Not absorbed | 3 000  | +    | 20 480   | —     |
|             | Absorbed     | 2 000  | +    | —  | —     |
| Protein A   | Not absorbed | 80   | +    | 20 480   | —     |
|             | Absorbed     | 80   | +    | —  | —     |
| Extract A   | Not absorbed | 10   | +    | 2 560  | 1 280 |
|             | Absorbed     | 10   | +    | —  | 320   |

STSC = Sensitized tanned sheep cells

SASC = Sensitized normal sheep cells

\* Polysaccharide A examined in Wood 46 serum, protein A and extract A in Cowan I serum

Our polysaccharide A preparation also sensitized tanned sheep cells (Table 2). Complete sensitization of 0.1 ml of packed sheep cells was obtained with 0.02 mg of polysaccharide A. The haemagglutinin titre in Wood 46 antiserum was 1:20,480 using standard conditions and even 1:40,960 when the erythrocyte concentration was diminished.

Wood 46 and tanned sheep cells from Wood 46 antiserum was also demonstrated in other strains of *Staph aureus*, e.g. in strain Cowan I, from which protein A is prepared.

The ability to sensitize normal sheep cells was destroyed at 100°C at alkaline pH but not at acid pH. It was resistant to trypsin (Table 4). These properties and the distribution of the antigen indicate that it is of the Rantz type (21).

The antigen sensitizing normal sheep cells could be separated from the precipitinogens protein A and polysaccharide A on ion exchange fractionation of crude extracts. An experiment was described in part I of this investigation (2), in which chromatography of extract A resulted in the separation of 3 fractions. One fraction contained only the antigen sensitizing normal sheep cells, the second fraction contained only an antigen sensitizing tanned sheep cells (see below), and the third fraction contained the precipitinogen (protein A) in addition to minor amounts of the two sensitizing substances. Chemical examination of the fraction containing only the antigen sensitizing normal sheep cells, showed that it contained ribose, glucuronic acid, and only small amounts of three amino acids.

Absorption of extract A with normal sheep cells, removed all antigen sensitizing normal sheep cells while the precipitin titre (protein A) and the ability to sensitize tanned sheep cells remained unchanged. The absorption experiments and the separation on DEAE cellulose column thus show that the antigen sensitizing normal sheep cells is not identical to any other antigen present.

### *Sensitization of Tanned Sheep Cells*

Extract A and protein A sensitized tanned sheep cells to agglutination both in Cowan I antiserum and in normal human serum. When 0.1 ml of packed, tanned erythrocytes were treated with 0.2 mg of protein A (or extract A), the haemagglutinin titre of Cowan I antiserum was 1:20,480 to 1:2,560 and that of normal human serum of the same order (Table 2). When extract A was absorbed with large doses of tanned erythrocytes, all antigen sensitizing tanned cells was removed while the antigen sensitizing normal cells and the precipitinogen were still present (Table 3). Absorption of Cowan I antiserum with tanned erythrocytes sensitized with extract A, removed the corresponding antibodies, but not antibodies against normal erythrocytes sensitized with extract A or against the precipitinogen. Thus the antigen sensitizing tanned sheep cells is not identical to the antigen in extract A which sensitizes normal cells, or to the precipitinogen (protein A). The antigen sensitizing tanned cells demonstrated in extract A and in protein A, were shown by absorption to be identical.

In the fractionation experiment on DEAE cellulose column reported in (2), the material sensitizing tanned cells could be separated from the two other antigens present in extract A. The separated material was only able to sensitize tanned sheep cells, and gave no precipitation on the ring test or on agar. The chemical examination of the fraction

showed that it contained 5 amino acids (Lys, Gly, Ser, Glu A. and Ala) and no sugar. The sensitizing ability was destroyed by trypsin (Table 4). These experiments thus confirm the results of the absorptions in that the antigen in extract A (and protein A) sensitizing tanned sheep cells is a distinct antigen. The antigen is of protein nature.

TABLE 2  
*Haemagglutination in Rabbit Immune Sera and Human Serum*

| Sensitize sheep cells | Haemagglutinin titres (reciprocal values) in sera |         |           |       |
|-----------------------|---|---------|-----------|-------|
|                       | Cowan I   | Wood 46 | Poly STSC | Human |
| Extract A STSC        | 2560  | 80      | 40        | 1280  |
| Extract A SASC        | 1280  | 160     | —         | —     |
| Protein A STSC        | 20480   | 80      | 40        | 10240 |
| Poly A STSC           | 80  | 20480   | 80        | 640   |

STSC = Sensitized tanned sheep cells

SASC = Sensitized normal sheep cells

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells

TABLE 3  
*Absorption of Preparations with Tanned Sheep Cells*

| Preparations |              | Precipitation in homologous serum <sup>a</sup> (reciprocal values) |      | Haemagglutination in homologous serum <sup>a</sup> (reciprocal values) |      |
|--------------|--------------|--|------|--|------|
|              |              | Ring test  | Agar | STSC   | SASC |
| Poly A       | Not absorbed | 3000   | +    | 20480  | —    |
|              | Absorbed     | 3000   | +    | —  | —    |
| Protein A    | Not absorbed | 80   | +    | 20480  | —    |
|              | Absorbed     | 80   | +    | —  | —    |
| Extract A    | Not absorbed | 10   | +    | 2560   | 1280 |
|              | Absorbed     | 10   | +    | —  | 320  |

STSC = Sensitized tanned sheep cells

SASC =

<sup>a</sup> Polysaccharide A

Cowan I

Our polysaccharide A preparation also sensitized tanned sheep cells (Table 2). Complete sensitization of 0.1 ml of packed sheep cells was obtained with 0.02 mg of polysaccharide A. The haemagglutinin titre in Wood 46 antiserum was 1:20480 using standard conditions and even 1:40960 when the erythrocyte concentration was reduced. The antigen in extract A and protein A is of protein nature and is prepared from strain Cowan I, from which protein A is prepared.



The ability to sensitize normal sheep cells was destroyed at 100° C at alkaline pH but not at acid pH. It was resistant to trypsin (Table 4). These properties and the distribution of the antigen indicate that it is of the Rantz type (21).

The antigen sensitizing normal sheep cells could be separated from the precipitinogens protein A and polysaccharide A on ion exchange fractionation of crude extracts. An experiment was described in part I of this investigation (2), in which chromatography of extract A resulted in the separation of 3 fractions. One fraction contained only the antigen sensitizing normal sheep cells, the second fraction contained only an antigen sensitizing tanned sheep cells (see below), and the third fraction contained the precipitinogen (protein A) in addition to minor amounts of the two sensitizing substances. Chemical examination of the fraction containing only the antigen sensitizing normal sheep cells showed that it contained ribose, glucuronic acid, and only small amounts of three amino acids.

Absorption of extract A with normal sheep cells, removed all antigen sensitizing normal sheep cells while the precipitin titre (protein A) and the ability to sensitize tanned sheep cells remained unchanged. The absorption experiments and the separation on DEAE cellulose column thus show that the antigen sensitizing normal sheep cells is not identical to any other antigen present.

### *Sensitization of Tanned Sheep Cells*

Extract A and protein A sensitized tanned sheep cells to agglutination both in Cowan I antiserum and in normal human serum. When 0.1 ml of packed, tanned erythrocytes were treated with 0.2 mg of protein A (or extract A), the haemagglutinin titre of Cowan I antiserum was 1:20,480 to 1:2,560 and that of normal human serum of the same order (Table 2). When extract A was absorbed with large doses of tanned erythrocytes, all antigen sensitizing tanned cells was removed while the antigen sensitizing normal cells and the precipitinogen were still present (Table 3). Absorption of Cowan I antiserum with tanned erythrocytes sensitized with extract A, removed the corresponding antibodies but not antibodies against normal erythrocytes sensitized with extract A or against the precipitinogen. Thus the antigen sensitizing tanned sheep cells is not identical to the antigen in extract A which sensitizes normal cells, or to the precipitinogen (protein A). The antigen sensitizing tanned cells demonstrated in extract A and in protein A, were shown by absorption to be identical.

In the fractionation experiment on DEAE cellulose column reported in (2), the material sensitizing tanned cells could be separated from the two other antigens present in extract A. The separated material was only able to sensitize tanned sheep cells, and gave no precipitation on the ring test or on agar. The chemical examination of the fraction

possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances. In the fraction containing the sensitizing antigen the same 5 amino acids were found as in the corresponding fraction obtained from extract A. The same amino acids were also demonstrated by Haukenes (4) in polysaccharide A, constituting the mucopeptide moiety of the material. The antigen in polysaccharide A sensitizing tanned cells was sensitive to trypsin whereas the precipitinogen (polysaccharide A) is resistant.

TABLE 5

*Haemagglutination after Cross Absorptions of Sera with Polysaccharide A STSC and Protein A STSC*

| Sensitized sheep cells  | Lewin's serum absorbed with |                | Wood's serum absorbed with |                | Lewin's A STSC serum* absorbed with |                |
|-------------------------|-----------------------------|----------------|----------------------------|----------------|-------------------------------------|----------------|
|                         | Polysaccharide A STSC       | Protein A STSC | Polysaccharide A STSC      | Protein A STSC | Polysaccharide A STSC               | Protein A STSC |
| 1 Polysaccharide A STSC | 1.80                        | —              | 1.80                       | —              | —                                   | 1.40           |
| Protein A STSC          | —                           | —              | —                          | —              | —                                   | —              |

STSC = Sensitized tanned sheep cells

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells. Serum titres before absorption are given in Table 2.

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutination reaction became negative. The supernatant was then analyzed for the presence of amino acids. The small amount of material made it difficult to obtain exact results, but it seemed that the amino acid content had been considerably reduced after absorption.

The tanned cell sensitizing antigens contaminating our protein A and polysaccharide A preparations can thus be separated from the precipitinogens. The latter have not yet been prepared free of the sensitizing antigens. As these sensitizing antigens are proteins, the polysaccharide A precipitinogen may be prepared free of the sensitizing antigen by enzymatic digestion. The protein A precipitinogen is however destroyed by trypsin.

In polysaccharide A the sensitizing antigen is undoubtedly attached to the mucopeptide moiety. *Staph aureus* H ribitol tetracholic acid has the same precipitating antigen as polysaccharide A but lacks the mucopeptide group (6). None of the *Staph aureus* ribitol tetracholic acids examined were able to sensitize tanned sheep cells.

## DISCUSSION

The significance of the double line appearing on agar precipitation of system A against the homologous whole rabbit immune serum but not against normal human serum has not been explained. It appears quite regularly and does not seem to be artificial. The double line has earlier

TABLE 4  
Tryptic Digestion of Preparations

| Preparations |             | Precipitation in homologous serum* |      | Haemagglutination in Cowan I serum |      |
|--------------|-------------|------------------------------------|------|------------------------------------|------|
|              |             | Ring test                          | Agar | STSC                               | SNSC |
| Poly A       | Not treated | +                                  | +    | +                                  | -    |
|              | Treated     | +                                  | +    | -                                  | -    |
| Protein A    | Not treated | +                                  | +    | +                                  | -    |
|              | Treated     | -                                  | -    | -                                  | -    |
| Extract A    | Not treated | +                                  | +    | +                                  | +    |
|              | Treated     | -                                  | -    | -                                  | +    |

STSC = Sensitized tanned sheep cells

SNSC = Sensitized normal sheep cells

\* Polysaccharide A precipitated in Wood 46 serum protein A and extract A in Cowan I serum

+ after tryptic digestion = titres unchanged

Haemagglutinins reacting with polysaccharide A sensitized tanned sheep cells were present in varying amounts in all our *Staph aureus* antisera, including serum Cowan I, and in normal human serum. These haemagglutinins were also present in antisera against 3 of the 4 strains of *Staph epidermidis* from which polysaccharide B and AC were prepared. The strains had been shown to contain small amounts of polysaccharide A (13).

Absorption of polysaccharide A with tanned sheep cells showed that its sensitizing antigen is not identical to the precipitinogen. The sensitizing substance could be completely removed, whereas the ring test titre and the precipitation line on agar remained unchanged (Table 3).

Cross-absorption experiments were performed to see whether the substance in protein A sensitizing tanned sheep cells is identical to that in polysaccharide A. As shown in Table 2, the two substances cross-react but the titres are much higher in the homologous sera than in the heterologous sera. The cross-absorptions (Table 5) showed that polysaccharide A sensitized tanned cells were able to exhaust the sera, whereas protein A sensitized tanned cells did not seem to remove the antibodies directed towards polysaccharide A sensitized tanned cells. The two substances sensitizing tanned sheep cells thus seem to be different serologically although they appear to be very similar chemically. This problem will be further investigated.

Rabbits were immunized with tanned sheep erythrocytes sensitized with polysaccharide A. After absorption of the sera with normal sheep cells, they agglutinated both polysaccharide A sensitized tanned cells and protein A sensitized tanned cells to low titres (Table 2). Precipitins against polysaccharide A or agglutinins toward whole, live staphylococci were not detected.

When polysaccharide A was run on a DEAE cellulose column, it was

possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances. In the fraction containing the sensitizing antigen, the same 5 amino acids were found as in the corresponding fraction obtained from extract A. The same amino acids were also demonstrated by Haukenes (4) in polysaccharide A, constituting the mucopeptide moiety of the material. The antigen in polysaccharide A sensitizing tanned cells, was sensitive to trypsin, whereas the precipitinogen (polysaccharide A) is resistant.

TABLE 5

*Haemagglutination after Cross Absorptions of Sera with Polysaccharide A STSC and Protein A STSC*

| Sensitize sheep cells | Cow 1 serum absorbed with |                | Wool 1 serum absorbed with |                | Poly A STSC serum* absorbed with |                |
|-----------------------|---------------------------|----------------|----------------------------|----------------|----------------------------------|----------------|
|                       | Poly A STSC               | Protein A STSC | Poly A STSC                | Protein A STSC | Poly A STSC                      | Protein A STSC |
| Poly A STSC           | -                         | 1.80           | -                          | 1.80           | -                                | 1.40           |
| Protein A STSC        | -                         | -              | -                          | -              | -                                | -              |

STSC = Sensitized tanned sheep cells

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells. Serum titres before absorption are given in Table 2.

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutination reaction became negative. The supernatant was then on 1:1000.

The tanned cell sensitizing antigens contaminating our protein A and polysaccharide A preparations can thus be separated from the precipitinogens. The latter have not yet been prepared free of the sensitizing antigens. As these sensitizing antigens are proteins, the polysaccharide A precipitinogen may be prepared free of the sensitizing antigen by enzymatic digestion. The protein A precipitinogen is, however, destroyed by trypsin.

In polysaccharide A the sensitizing antigen is undoubtedly attached to the mucopeptide moiety. *Staph aureus* H ribitol teichoic acid has the same precipitating antigen as polysaccharide A but lacks the mucopeptide group (6). None of the *Staph aureus* ribitol teichoic acids examined were able to sensitize tanned sheep cells.

## DISCUSSION

The significance of the double line appearing on agar precipitation of protein A against the homologous whole, rabbit immune serum, against non-sensitized cells, and against sensitized cells, is not yet regulated.

TABLE 4  
*Tryptic Digestion of Preparations*

| Preparations |             | Precipitation in homologous serum* |      | Haemagglutination in Cowan I serum |      |
|--------------|-------------|------------------------------------|------|------------------------------------|------|
|              |             | Ring test                          | Agar | STSC                               | SNSC |
| Poly A       | Not treated | +                                  | +    | +                                  | -    |
|              | Treated     | +                                  | +    | -                                  | -    |
| Protein A    | Not treated | +                                  | +    | +                                  | -    |
|              | Treated     | -                                  | -    | -                                  | -    |
| Extract A    | Not treated | +                                  | +    | +                                  | +    |
|              | Treated     | -                                  | -    | -                                  | +    |

STSC = Sensitized tanned sheep cells

SNSC = Sensitized normal sheep cells

\* Polysaccharide A precipitated in Wood 46 serum protein A and extract A in Cowan I serum

+ after tryptic digestion = titres unchanged

Haemagglutinins reacting with polysaccharide A sensitized tanned sheep cells were present in varying amounts in all our *Staph aureus* antisera, including serum Cowan I, and in normal human serum. These haemagglutinins were also present in antisera against 3 of the 4 strains of *Staph epidermidis* from which polysaccharide B and AC were prepared. The strains had been shown to contain small amounts of polysaccharide A (13).

Absorption of polysaccharide A with tanned sheep cells showed that its sensitizing antigen is not identical to the precipitinogen. The sensitizing substance could be completely removed, whereas the ring test titre and the precipitation line on agar remained unchanged (Table 3).

Cross-absorption experiments were performed to see whether the substance in protein A sensitizing tanned sheep cells is identical to that in polysaccharide A. As shown in Table 2, the two substances cross-react but the titres are much higher in the homologous sera than in the heterologous sera. The cross-absorptions (Table 3) showed that polysaccharide A sensitized tanned cells were able to exhaust the sera, whereas protein A sensitized tanned cells did not seem to remove the antibodies directed towards polysaccharide A sensitized tanned cells. The two substances sensitizing tanned sheep cells thus seem to be different serologically although they appear to be very similar chemically. This problem will be further investigated.

Rabbits were immunized with tanned sheep erythrocytes sensitized with polysaccharide A. After absorption of the sera with normal sheep cells, they agglutinated both polysaccharide A sensitized tanned cells and protein A sensitized tanned cells to low titres (Table 2). Precipitins against polysaccharide A or agglutinins toward whole, live staphylococci were not detected.

When polysaccharide A was run on a DEAE-cellulose column, it was

possible to separate the antigen sensitizing tanned cells from the mixture of sensitizing and precipitating substances. In the fraction containing the sensitizing antigen, the same 5 amino acids were found as in the corresponding fraction obtained from extract A. The same amino acids were also demonstrated by Haukenes (4) in polysaccharide A constituting the mucopeptide moiety of the material. The antigen in polysaccharide A sensitizing tanned cells was sensitive to trypsin, whereas the precipitinogen (polysaccharide A) is resistant.

TABLE 5

*Haem agglutination after Cross Absorption of Sera with Polysaccharide A STSC and Protein A STSC*

| Sensitized sheep cells | Goat serum absorbed with |                | Woolf serum absorbed with |                | Pol. A STSC serum* absorbed with |                |
|------------------------|--------------------------|----------------|---------------------------|----------------|----------------------------------|----------------|
|                        | Pol. A STSC              | Protein A STSC | Pol. A STSC               | Protein A STSC | Pol. A STSC                      | Protein A STSC |
| Pol. A STSC            |                          | 1:80           |                           | 1:80           |                                  | 1:40           |
| Protein A STSC         | —                        | —              |                           |                | —                                | —              |

STSC = Sensitized tanned sheep cells.

\* Rabbit immune serum against polysaccharide A sensitized tanned sheep cells. Serum titres before absorption are given in Table 2.

A sample of polysaccharide A was absorbed with tanned sheep cells until the haemagglutination reaction became negative. The supernatant was then analyzed for the presence of amino acids. The small amount of material made it difficult to obtain exact results, but it seemed that the amino acid content had been considerably reduced after absorption.

The tanned cell sensitizing antigens contaminating our protein A and polysaccharide A preparations can thus be separated from the precipitinogens. The latter have not yet been prepared free of the sensitizing antigens. As these sensitizing antigens are proteins, the polysaccharide A precipitinogen may be prepared free of the sensitizing antigen by enzymatic digestion. The protein A precipitinogen is, however, destroyed by trypsin.

In polysaccharide A the sensitizing antigen is undoubtedly attached to the mucopeptide moiety. *Staph. aureus* H ribitol teichoic acid has the same precipitating antigen as polysaccharide A but lacks the mucopeptide group (6). None of the *Staph. aureus* ribitol teichoic acids examined were able to sensitize tanned sheep cells.

## DISCUSSION

The significance of the double line appearing on agar precipitation of protein A against the homologous whole rabbit immune serum is argued as follows:

TABLE 4  
*Tryptic Digestion of Preparations*

| Preparations |             | Precipitation<br>in homologous serum* |      | Haemagglutination<br>in Cowan I serum |      |
|--------------|-------------|---------------------------------------|------|---------------------------------------|------|
|              |             | Ring test                             | Agar | STSC                                  | SNSC |
| Poly A       | Not treated | +                                     | +    | +                                     | -    |
|              | Treated     | +                                     | +    | -                                     | -    |
| Protein A    | Not treated | +                                     | +    | +                                     | -    |
|              | Treated     | -                                     | -    | -                                     | -    |
| Extract A    | Not treated | +                                     | +    | +                                     | +    |
|              | Treated     | -                                     | -    | -                                     | +    |

STSC = Sensitized tanned sheep cells

SNSC = Sensitized normal sheep cells

\* Polysaccharide A precipitated in Wood 46 serum protein A and extract A in Cowan I serum

+ after tryptic digestion = titres unchanged

Haemagglutinins reacting with polysaccharide A sensitized tanned sheep cells were present in varying amounts in all our *Staph aureus* antisera, including serum Cowan I, and in normal human serum. These haemagglutinins were also present in antisera against 3 of the 4 strains of *Staph epidermidis* from which polysaccharide B and AC were prepared. The strains had been shown to contain small amounts of polysaccharide A (13).

Absorption of polysaccharide A with tanned sheep cells showed that its sensitizing antigen is not identical to the precipitinogen. The sensitizing substance could be completely removed, whereas the ring test titre and the precipitation line on agar remained unchanged (Table 3).

Cross-absorption experiments were performed to see whether the substance in protein A sensitizing tanned sheep cells is identical to that in polysaccharide A. As shown in Table 2, the two substances cross-react but the titres are much higher in the homologous sera than in the heterologous sera. The cross-absorptions (Table 3) showed that polysaccharide A sensitized tanned cells were able to exhaust the sera, whereas protein A sensitized tanned cells did not seem to remove the antibodies directed towards polysaccharide A sensitized tanned cells. The two substances sensitizing tanned sheep cells thus seem to be different serologically although they appear to be very similar chemically. This problem will be further investigated.

Rabbits were immunized with tanned sheep erythrocytes sensitized with polysaccharide A. After absorption of the sera with normal sheep cells, they agglutinated both polysaccharide A sensitized tanned cells and protein A sensitized tanned cells to low titres (Table 2). Precipitins against polysaccharide A or agglutinins toward whole, live staphylococci were not detected.

When polysaccharide A was run on a DEAE cellulose column, it was

sensitizing tanned cells can be separated from the precipitinogen on the ion exchange column it seems to be quite firmly attached to the teichoic acid. The sensitizing antigen may therefore have the same distribution as polysaccharide A (teichoic acid) i.e. be present in practically all *Staph aureus* strains. This may explain why the sensitizing antigen has also been demonstrated in certain strains of *Staph epidermidis* which according to *Iosnegard & Oeding* (13) in addition to polysaccharide B and C also have small amounts of polysaccharide A. The distribution in staphylococci of antigens sensitizing tanned erythrocytes should be more thoroughly investigated.

*Morse* (15) reported that the teichoic acid isolated by him from the wall of one strain of *Staph aureus* sensitized tanned sheep cells and that  $\beta$  linked N acetylglucosamine was responsible for this activity. We found that the specific line on agar given by *Staph aureus* II teichoic acid and polysaccharide A was due to the  $\beta$  linked N acetylglucosamine (6). The teichoic acid preparations examined by us were however not able to sensitize erythrocytes. Further our tanned cells sensitizing substance was separated from the precipitinogen on the ion exchange column and shown to be composed of 5 amino acids. *Morse* used a 100 times higher sensitizing dose than we did and it is probably that the sensitizing ability of his teichoic acid preparation is due to contamination with the sensitizing protein antigen.

#### SUMMARY

Purified protein A (antigen A) has been examined serologically. On agar precipitation a single line developed with normal human serum whereas a double line was regularly observed with rabbit immune serum.

An antigen sensitizing tanned sheep erythrocytes to agglutination in rabbit immune serum and in normal human serum was demonstrated in protein A. A similar antigen was present in polysaccharide A but not in wall teichoic acid from *Staph aureus*. In polysaccharide A and

in tanned cell sensitizing antigens seem to be present in most strains of *Staph aureus* and in certain strains of *Staph epidermidis*.

An antigen sensitizing normal sheep erythrocytes to agglutination

Protein A purified polysaccharide A or *Staph aureus* wall teichoic acid. It was isolated from extract A on the ion exchange column and contained glucuronic acid and ribose.



been described by *Oeding & Haukenes* (20). It may be due to small molecular differences in the protein A antigen which stimulate the formation of two corresponding antibodies. As it has not been possible to separate two substances corresponding to the lines, they must have very similar properties. The antibody or antibodies in Cowan I antiserum responsible for the double line on agar, may therefore be the complete antibody response to the antigen, whereas the antibody in normal human serum may be incomplete. This may be of interest in connection with the rather peculiar fact that all normal human sera contain this antibody.

It is also interesting that normal human sera seem to contain antibodies in high titres directed against the antigens in *Staph aureus* sensitizing tanned sheep cells. We were not able to demonstrate antibodies in a limited number of normal human sera against the antigen sensitizing normal sheep cells. Antibodies against presumably the same substance have, however, been found in normal human sera by others (22, 21, 16).

The antigen in extract A sensitizing normal sheep cells has the properties of the Rantz antigen (21). It was demonstrated in varying amounts in strains of *Staph aureus*, in *Staph epidermidis* and *B subtilis*, but not in *M lysodeikticus*. Further it is sensitive to heat at alkaline but not at acid pH. The antigen is undoubtedly identical to that described by *Jensen et al* (11) in extract A. There does not seem to be more than one substance in *Staph aureus* sensitizing normal erythrocytes, although this possibility has not been systematically investigated. It is, therefore, likely that the sensitizing substances described by other authors (22, 19, 5) are identical to that described here.

The antigen sensitizing normal sheep cells is not identical to any of the other antigens present in extract A. This was shown by absorption of the extract and antiserum, and by fractionation on ion exchange column. The antigen seems to be quite loosely attached to the other antigenic substances in extract A, as it could easily be isolated on the column. It is therefore not present in the purified protein A or polysaccharide A preparations. As it is not destroyed by trypsin and shown to contain glucuronic acid and ribose, the antigen is apparently a sugar. *Gorzynski et al* (1) suggest that the Rantz antigen may be related to teichoic acid. Our experiments did not support this theory. First, the teichoic acid preparations examined and polysaccharide A had not the ability to sensitize normal sheep cells. Second, the isolated antigen sensitizing normal cells was not a teichoic acid.

The antigens sensitizing tanned sheep cells appear to be widely distributed in *Staph aureus* strains. In polysaccharide A the antigen seems to be bound to the mucopeptide moiety contaminating the precipitinogen (polysaccharide A), which is serologically identical to the wall teichoic acid of *Staph aureus* H. The teichoic acid preparations examined, had no sensitizing antigen. Although the substance in polysaccharide A

sensitizing tanned cells can be separated from the precipitinogen on the ion exchange column it seems to be quite firmly attached to the teichoic acid. The sensitizing antigen may therefore have the same distribution as polysaccharide A (teichoic acid) i.e. be present in practically all *Staph aureus* strains. This may explain why the sensitizing antigen has also been demonstrated in certain strains of *Staph epidermidis*, which according to *Losnevard & Oedin* (13) in addition to polysaccharide B and C also have small amounts of polysaccharide A. The distribution in staphylococci of antigens sensitizing tanned erythrocytes should be more thoroughly investigated.

*Morse* (15) reported that the teichoic acid isolated by him from the wall of one strain of *Staph aureus* sensitized tanned sheep cells and that  $\beta$  linked N acetylglucosamine was responsible for this activity. We found that the specific line on agar given by *Staph aureus* H teichoic acid and polysaccharide A was due to the  $\beta$  linked N acetylglucosamine (6). The teichoic acid preparations examined by us were however not able to sensitize erythrocytes. Further our tanned cells sensitizing substances was separated from the precipitinogen on the ion exchange column and shown to be composed of 5 amino acids. *Morse* used a 100 times higher sensitizing dose than we did and it is probably that the sensitizing ability of his teichoic acid preparation is due to contamination with the sensitizing protein antigen.

#### SUMMARY

Purified  $\gamma$  protein A (antigen A) has been examined serologically. On agar precipitation a single line developed with normal human serum whereas a double line was regularly observed with rabbit immune serum.

An antigen sensitizing tanned sheep erythrocytes to agglutination in rabbit immune serum and in normal human serum was demonstrated in  $\gamma$  protein A. A similar antigen was present in polysaccharide A but not in wall teichoic acid from *Staph aureus*. In polysaccharide A the mucopolysaccharide group is apparently responsible for the sensitizing activity. The antigens in  $\gamma$  protein A and polysaccharide A sensitizing tanned cells were isolated on ion exchange columns and contained 5 amino acids. Tanned cell sensitizing antigens seem to be present in most strains of *Staph aureus* and in certain strains of *Staph epidermidis*.

An antigen sensitizing normal sheep erythrocytes was found in washings and crude extracts of *Staph aureus* strains and in strains of *Staph epidermidis* and *B subtilis* but not in *M lysodeikticus*. This antigen is apparently of the Rantz type. The antigen is not present in purified  $\gamma$  protein A, purified polysaccharide A or *Staph aureus* wall teichoic acid. It was isolated from extract A on the ion exchange column and contained glucuronic acid and ribose.

been described by *Oeding & Haukenes* (20) It may be due to small molecular differences in the protein A antigen which stimulate the formation of two corresponding antibodies As it has not been possible to separate two substances corresponding to the lines, they must have very similar properties The antibody or antibodies in Cowan I antiserum responsible for the double line on agar, may therefore be the complete antibody response to the antigen, whereas the antibody in normal human serum may be incomplete This may be of interest in connection with the rather peculiar fact that all normal human sera contain this antibody

It is also interesting that normal human sera seem to contain antibodies in high titres directed against the antigens in *Staph aureus* sensitizing tanned sheep cells We were not able to demonstrate antibodies in a limited number of normal human sera against the antigen sensitizing normal sheep cells Antibodies against presumably the same substance have, however, been found in normal human sera by others (22, 21, 16)

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The precipitinogens protein A and polysaccharide A and the sensitizing substances were shown on absorption and column fractionation to be different antigens

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serology, immunobiological characteristics, and pathogenicity in animals. It was temporarily called *Pasteurella pseudotuberculosis*  $\lambda$  (Knapp & Thal). Strains have been isolated in Holland, West Germany, Switzerland, and Denmark, always from animals, principally chinchilla and hare, and in one instance from a dog.

In the case about to be described, *Past pseudotuberculosis*  $\lambda$  was isolated from man. The only previous examples of this are the two cases published by Hassig in 1949.

### CASE HISTORY

A 21 year old man previously well was admitted to the surgical clinic with a history of 24 hours of abdominal pain. The pain started in the epigastrium but had migrated down to the right iliac fossa. There was nausea but no vomiting. Stool was normal. No diarrhoea had recently been noted. The patient denied contact with animals. The temperature on admittance was  $37.7^{\circ}\text{C}$  and the white count 12,600. There was distinct tenderness on palpation over McBurney's point but no muscular defence nor a palpable mass was noted. No tenderness was found at rectal examination.

One of the inflamed lymph nodes was extirpated and halved. One half was sent using sterile precautions for bacteriological and the other for pathological examination.

The postoperative course was quite uneventful. No antibiotics were administered. The patient was discharged from hospital on the third postoperative day. The wound healed primarily in a week and he resumed work after four weeks. He had since had no abdominal complaints. Four and a half months postoperatively, a small bowel series using barium contrast showed a slight narrowing of the distal five cms of the ileum. However a repeat examination six months postoperatively was normal.

### Histo-Pathology

The appendix was swollen and reddened only in the proximal part and had a persisting lumen. Histologically, the mucosa was largely preserved, but with abundant lymphoid tissue with big reaction centres. In the proximal part it showed patches of oval or irregular necrosis with closely packed polymorphonuclear leucocytes and nuclear debris. A few small vessels contained fibrin thrombi. In the surroundings there were fairly numerous plasma cells, some reticulocytes and single eosinophilic leucocytes but no giant cells or granulomas. No lesions were found in the distal part of the appendix. The submucosa was oedematous with streaks of round cells and leucocytes, the inflammatory reaction extending to the serosa, which was partly covered with fibrin.

The Institute of Clinical Bacteriology (Head: Professor S. Winkblad) The Department of Surgery (Head: Professor H. Wulff) and The Institute of Pathology (Head: Professor F. Linell) Malmö General Hospital University of Lund, Sweden

## A CASE OF HUMAN INFECTION WITH *PASTEURELLA PSEUDOTUBERCULOSIS* A

By

M. G. CARLSSON, H. RYD and N. H. STERNBY

Received 1963 III

Since the end of the last century *Pasteurella pseudotuberculosis* has been known to cause infections in animals, especially rodents. The term pseudotuberculosis was created by the pathologist C. J. F. Berth who described the illness in guinea pigs and rabbits in 1883-86. The bacterium was isolated by Malassez & Vignal at the same time in 1884.

From the first half of the twentieth century only some twenty human cases have been reported. In 1949 Hassig made a summary review of these while presenting two cases of his own. Most of the cases were septic typhoid-like conditions and the mortality was high. In 1952 Puchaud described a case resembling appendicitis with mesenteric lymphadenitis from which *Pasteurella pseudotuberculosis* was isolated. Among the cases of acute abdomen that Masshoff described in 1953 he set apart one abscess-forming, reticulocytic mesenteric lymphadenopathy in the ileocecal angle presenting a clinical picture indistinguishable from that of acute appendicitis. Further investigations by Knapp & Masshoff showed the condition to be associated with the presence of *Past. pseudotuberculosis* (1954). Other clinical manifestations such as subacute or chronic appendicitis, enteritis and gastroenteritis were described. In some instances it was possible to isolate the organism but more often the diagnosis was established by clinical, serological and histological examinations.

A number of reviews and reports show that the infection is not uncommon in man (Knapp 1959, Mair et al. 1960, Arnulf 1960, Mollaret 1960, Schmidt 1960, Beerens 1960, Kjaer 1960, Daniels 1961, Randall & Mair 1962, Boeltger 1962, Fezler 1962).

However, only a small number of non-specific infections in the ileocecal region can be referred to *Past. pseudotuberculosis*. In most cases the aetiology remains unknown.

In 1961 Daniels described a bacterium isolated from chinchillas and resembling *Past. pseudotuberculosis*. Its morphological and cultural properties were compatible with *Past. pseudotuberculosis* but it differed from the previously known groups I-V with regard to biochemistry.

serology, immunobiological characteristics, and pathogenicity in animals. It was temporarily called *Pasteurella pseudotuberculosis* A (Knapp & Thal). Strains have been isolated in Holland, West Germany, Switzerland and Denmark, always from animals, principally chinchilla and hare and in one instance from a dog.

In the case about to be described *Past pseudotuberculosis* A was isolated from man. The only previous examples of this are the two cases published by Hussig in 1949.

### CASE HISTORY

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Acute appendicitis being suspected an acute laparotomy was done. The appendix was found free and mobile. 2-3 cms of its base was inflamed, swollen and firm whereas its distal part was thin and pale and without signs of inflammation. There was minimal extension of the inflammation to the adjacent caecum which was otherwise normal. The terminal 10 cms of the ileum was reddened, thickened

using sterile precautions for bacteriological and the other for pathological examination.

The postoperative course was quite uneventful. No antibiotics were administered. The patient was discharged from hospital on the third postoperative day. The wound healed primarily in a week and he resumed work after four weeks. He had since had no abdominal complaints. Four and a half months postoperatively a small bowel series using barium contrast showed a slight narrowing of the distal five cms of the ileum. However a repeat examination six months postoperatively was normal.

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## A CASE OF HUMAN INFECTION WITH PASTEURELLA PSEUDOTUBERCULOSIS X

By

M G CARLSSON, H RYD and N H STERNBY

Received 25 II 64

Since the end of the last century, *Pasteurella pseudotuberculosis* has been known to cause infections in animals, especially rodents. The term pseudotuberculosis was created by the pathologist C J Eberth, who described the illness in guinea-pigs and rabbits in 1885-86. The bacterium was isolated by Malassez & Vignal at the same time in 1884.

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12th 1/160 May 28th 1/20 It was thus not possible to demonstrate an O antigen common to the strain of the patient and to *Past pseudotuberculosis* I-V. In the course of his illness however the patient formed immune bodies against the O antigen of his own strain.

Using the term strain 897 Winblad Knapp & Thal (1963) studied the relations between the *Pasteurella* strain of the patient and the strain isolated from chinchillas (Daniels 1962). They found that the two strains isolated from human material were identical and that they had the cultural biochemical properties characteristic of those strains that had been isolated from animals. *Pasteurella* phages did not attack any *Pasteurella* \ strain. Further details of these matters and the differential diagnosis between *Past pseudotuberculosis* I-V and *Pasteurella* \ are given in the article of Knapp & Thal.

#### SUMMARY

In one case of acute terminal ileitis in man spreading to the appendix and mesenteric lymph nodes a bacterium resembling *Pasteurella pseudotuberculosis* could be demonstrated. It proved identical (Knapp & Thal) with a strain isolated in 1949 (Hassig) from 2 patients suffering from septical typhoidal lethal conditions and it had the same cultural biochemical properties as *Pasteurella* \ strain isolated from animals. The histopathological picture of a mesenteric lymph node was non specific whereas changes occurring in the lymphoid tissue of the appendix showed some similarity to those described by Masshoff (1963). The clinical symptoms corresponded to an acute appendicitis and no complications occurred. In the patient's serum there was a significantly rising and then subsiding titre of O agglutinins against the bacterium which was found to have no O antigen in common with *Pasteurella pseudotuberculosis* strains I-V.

#### ADDENDUM

After this article had been finished one more case with positive serology was observed. A man 39 years old was taken ill on Nov 14th with symptoms indicating acute appendicitis. On appendectomy Nov 16th the appendix was found to be normal whereas the terminal part of the ileum was thickened and of a strong red colour. Only small mesenteric lymph nodes were seen. Histological examination was not done. On Nov 22nd O antigen of *Past pseudotuberculosis* \ was agglutinated by the patient's serum in dilution 1/160.

The lymph node was the size of a bean, with homogenous grey cut surface. Histologically the structure was well-preserved. The sinuses were often widened with swollen endothelial cells and contained plasma cells and leucocytes. There were no proliferating reticular cells, granulomas, giant cells, or necrosis.

The pathological-anatomical examination thus showed no specific lymphoglandular changes, but in the proximal part of the mucosa of the appendix there were patchy necroses. These changes differ from those described as typical of acute mesenteric lymphadenitis (Mass-hoff). In the latter, round or starshaped necroses crammed with leucocytes are usually seen, surrounded by a zone of reticular cells, and also uncharacteristic giant cells are sometimes seen. The changes occur mainly in lymph nodes and only seldom in the mucosa of the appendix. In our case, conditions were reversed.

### Bacteriology

Material from a mesenteric lymph node was enriched with peptone and glucose in ordinary placental broth at 37° C for 48 hours. Blood agar plates were inoculated and incubated aerobically both at 37° C and 22° C. As early as 48 hours later, there was a rich growth of yellow-grey, matt colonies, partly with a smooth and partly with a nodular surface and sharp edge. In Gram stained smears from the colonies, small slender, sometimes polestained Gram negative rods were seen, showing a moderate degree of pleomorphism in older colonies. On cultivation in U-tubes with semi-solid agar, the bacterium was motile at 22° C, but non-motile at 37° C. Its cultural and morphological characteristics were consequently those of *Pasteurella pseudotuberculosis*.

The bacterium rapidly produced acid but no gas with mannitol, saccharose, dextrose, and levulose, and more slowly with xylose, maltose, arabinose, and galactose. The indole, H<sub>2</sub>S, and oxidise reactions were negative, the nitrate and catalase reactions positive. Urease was produced on LSU agar (Juhlin & Ericson 1961). Lactose, inositol, rhamnose, raffinose, inulin, aesculin, and salicin were not influenced. In producing acid with saccharose, but not with rhamnose or aesculin, the strain differed from *Past. pseudotuberculosis*. In vitro, using disc technique, the strain proved insusceptible to sulphonamides, penicillin, and erythromycin, slightly sensitive to kanamycin, and sensitive to streptomycin, tetracyclines and chloramphenicol.

### Serology

O antisera from rabbits immunized with *Past. pseudotuberculosis* I-V did not agglutinate O antigen prepared from the strain of this patient. The patient's serum did not agglutinate O antigen belonging to pasteurilla strains I-III. The patient's own O antigen, however, was agglutinated by his serum, showing the following titres: Jan 15th 0, Febr

12th 1160 May 28th 120 It was thus not possible to demonstrate an O antigen common to the strain of the patient and to *Past pseudotuberculosis* I \ In the course of his illness however the patient formed immune bodies against the O antigen of his own strain

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# ROLE OF INTERFERON IN THE AUTOINTERFERENCE OF NEWCASTLE DISEASE VIRUS (NDV)

By

SVANTE HERMUDSSON

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In titrations especially of myxo and arboviruses it is a common observation that higher concentrations of virus induce less cellular damage and formation of less virus than lower virus concentrations. This phenomenon has been called autointerference, zone phenomenon or autoinhibition and has been ascribed to the presence of interferon or incomplete virus in the virus suspensions (for references see 14).

In a previous communication (12) it was shown that the autointerference of NDV in calf cultures can be suppressed by a concurrent infection with paramfluenza virus type 3. This effect appeared to be due to suppression of an interferon like inhibitor. The present study was undertaken in order to characterize the inhibitor and to establish its role in the autointerference of NDV in calf kidney cultures.

## MATERIALS AND METHODS

*Cell cultures* Primary calf kidney tissue of newborn kidney tissue of newborn roller tubes or plastic with 10 per cent lact (100 U penicillin/ml)

immunization of rabbits with  
The serum had a haemag  
of infectious virus and was stored at

**Virus titration** Infectivity was as a rule determined in roller tube cultures of calf kidney cells. Each of 5 tubes was inoculated with 0.1 ml of serial tenfold dilutions of the virus samples. The cultures were examined for cytopathic changes and haemadsorbing capacity after incubation for 5 to 7 days. The haemadsorption test was carried out according to the modification of Chanock *et al.* (3). The number of TC<sub>50</sub> was calculated by Karber's method.

**Plaque assay of NDV** was performed on plastic petri dishes with confluent sheets of HeLa cells. After washing with phosphate buffered saline (PBS) the plates were inoculated with 0.1 ml of tenfold dilutions of the samples. After an adsorption period of 30 minutes at 37° C the plates were overlaid with 3 ml MEM containing 10 per cent horse serum and 0.95 per cent agar (Special agar Noble Difco). The plates were stained after incubation for 2 days with 2 ml agar overlay containing neutral red in a final concentration of 1/20 000. The plaques were counted on the 4th day of incubation.

**Plaque assay of bovine enterovirus** was performed similarly on calf kidney cells grown in prescription bottles. The overlay for these cells was Farle's salt solution with 2.2 g NaHCO<sub>3</sub> per litre, 0.5 per cent bovalbumin hydrolysate, 3 per cent calf serum and 0.95 per cent agar.

**HA titrations** were performed in tubes by the pattern test. Serial twofold dilutions of the virus material in 0.15 M NaCl were mixed with an equal volume (0.5 ml) of a 0.5 per cent suspension of guinea pig erythrocytes. The tests were read after 2 hours at 4° C and the titre was expressed as the reciprocal of the highest initial dilution giving partial agglutination.

**Assay for interferon activity** Prior to titration the samples were centrifuged at 80 000 g for 2 hours. Serial twofold dilutions of the supernatants were then made in maintenance medium containing NDV antiserum at a final dilution of 1/80. The dilutions were incubated at 37° C for 2 hours and inoculated into roller tubes. Each dilution was inoculated into 2 tubes and each tube received 1 ml medium. After incubation for 18 to 24 hours the cultures were exposed to a challenge infection with 10<sup>4</sup> TC<sub>50</sub> of Sendai virus. The cultures were incubated for another 3 to 4 days and were subsequently examined for cytopathic changes and haemagglutinating activity. The interferon titre was expressed as the reciprocal of the highest dilution giving at least a fourfold reduction of the HA titre compared with the controls.

**Staining with fluorescent antibodies** Subcultures of calf kidney cells grown on coverslips in 16-ounce tubes were washed in PBS, fixed in acetone and air dried. Staining with fluorescent antibodies was then performed by the indirect method (25) with NDV antiserum diluted 1:16 in PBS and with goat antirabbit globulin conjugated with fluorescein isothiocyanate (Microbiological Associates, Bethesda, Maryland). The coverslips were mounted on object glasses in glycerol phosphate buffer and examined in a Zeiss fluorescence microscope equipped with a darkfield condenser and a mercury lamp (Osram HBO 200). The primary filter was BG 12 and the secondary Zeiss 50 alone or in combination with Zeiss 47.

**Other procedures** In order to inactivate interferon the virus suspensions were treated at 37° C for 2 hours with 0.5 mg of crystallized trypsin (Worthington Biochem Corp., New Jersey) per ml. The enzyme was inactivated by addition of 1 mg of soybean trypsin inhibitor (Mann Research Lab., New York) per ml. Treatments with DNase and RNase (Worthington Biochem Corp., New Jersey) were performed at 37° C for 30 minutes with the enzymes in concentrations of 100 µg per ml.

The ether sensitivity of the inhibitor was tested by shaking the inhibitory fluid with 20 per cent ether for 30 minutes at 4° C. The ether was removed by evaporating overnight at 4° C and then bubbling nitrogen gas through.

0.07 M HCl-KCl buffer  
adjusted to pH 7.4 by further  
maintenance medium

for 12 hours

## RESULTS

### *Demonstration of Autointerference*

An inhibited virus multiplication was regularly observed in calf kidney cultures inoculated with undiluted suspensions of NDV. Fig. 1

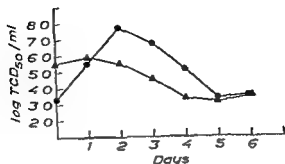


Fig 1

Growth curves of NDV in flask cultures of calf kidney cells, ▲—▲ inoculated with undiluted virus suspension ●—● virus diluted 10<sup>-2</sup>.

shows the virus yields in flask cultures inoculated either with 1 ml of the undiluted stock suspension or with 1 ml of the stock diluted 10<sup>-2</sup>, corresponding to input multiplicities of approximately 1 and 0.01 respectively. The medium was changed daily and assayed for infectivity and haemagglutinin. Before titration one part of the harvest was subjected to ultrasonic vibration (11) with the idea that NDV like several other paramyxoviruses (11, 21) produces haemagglutinin which can be unmasked by this treatment.

TABLE 1

*Production of Haemagglutinin in Calf Kidney Cultures Inoculated with Different Amounts of NDV*

| Dilution of NDV inoculated | HA units/ml culture fluid at indicated days after inoculation of NDV |         |         |         |         |         |
|----------------------------|--|---------|---------|---------|---------|---------|
|                            | 1  | 2       | 3       | 4       | 5       | 6       |
| 10 <sup>0</sup>            | <2 (2)*  | 2 (4)   | <2 (<2) | <2 (<2) | <2 (<2) | <2 (<2) |
| 10 <sup>-2</sup>           | <2 (<2)  | 32 (64) | 32 (64) | <2 (<2) | <2 (<2) | <2 (<2) |

\* Figures in brackets refer to titres after ultrasonic treatment

As shown in Fig 1 and Table 1 the production of infectious virus and haemagglutinin was significantly suppressed in the cultures inoculated with undiluted virus suspensions. Such cultures continuously produced low titres of infectious virus for a long period (Fig 2). A persistence of the NDV infection also occurred in secondary cultures prepared by trypsinization of primary cultures on day 2 after virus inoculation (Fig 2).

The content of viral antigen in the cells was studied by the fluorescent antibody technique. Monolayers of calf kidney cells grown on coverslips were infected with NDV at input multiplicities of approximately 5 and 0.05 respectively. At intervals after inoculation the cells were fixed in acetone and the media assayed for haemagglutinating



**Virus titration** Infectivity was as a rule determined in roller tube cultures of calf kidney cells. Each of 5 tubes was inoculated with 0.1 ml of serial tenfold dilutions of the virus samples. The cultures were examined for cytopathic changes and haemadsorbing capacity after incubation for 5 to 7 days. The haemadsorption test was carried out according to the modification of Chanock *et al.* (3). The number of TC<sub>50</sub> was calculated by Harber's method.

**Plaque assay of NDV** was performed on plastic petri dishes with confluent sheets of HeLa cells. After washing with phosphate buffered saline (PBS) the plates were inoculated with 0.1 ml of tenfold dilutions of the samples. After an adsorption period of 30 minutes at 37°C the plates were overlaid with 5 ml MEM containing 10 per cent horse serum and 0.5 per cent agar (Special agar Noble, Difco). The plates were stained after incubation for 2 days with 2 ml agar overlay containing neutral red in a final concentration of 1/20 000. The plaques were counted on the 4th day of incubation.

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The ether sensitivity of the inhibitor was tested by shaking the inhibitory fluid with 20 per cent ether for 30 minutes at 4°C. The ether was removed by evaporating overnight at 4°C and then bubbling nitrogen gas through.

Dialysis at pH 2.0 was performed in cellulose casings against 0.07 M HCl, HCl buffer for 18 hours at 4°C. The pH of the dialysate fluid was restored to pH 7.4 by further dialysis against 1 M NaCl solution for 12 hours and against maintenance medium for 12 hours.

## RESULTS

### Demonstration of Autointerference

An inhibited virus multiplication was regularly observed in calf kidney cultures inoculated with undiluted suspensions of NDV. Fig 1

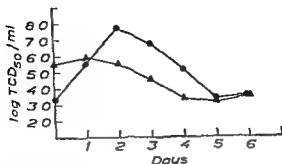


Fig 1

Growth curves of NDV in flask cultures of calf kidney cells ▲—▲ inoculated with undiluted virus suspension ●—● virus diluted  $10^2$

shows the virus yields in flask cultures inoculated either with 1 ml of the undiluted stock suspension or with 1 ml of the stock diluted  $10^2$ , corresponding to input multiplicities of approximately 1 and 0.01 respectively. The medium was changed daily and assayed for infectivity and haemagglutinin. Before titration one part of the harvest was subjected to ultrasonic vibration (11) with the idea that NDV like several other paramyxoviruses (11, 21) produces haemagglutinin which can be unmasked by this treatment.

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|                            | 1  | 2       | 3       | 4       | 5       | 6       |
| $10^0$                     | <2 (2)*  | 2 (4)   | <2 (<2) | <2 (<2) | <2 (<2) | <2 (<2) |
| $10^{-2}$                  | <2 (<2)  | 32 (64) | 32 (64) | <2 (<2) | <2 (<2) | <2 (<2) |

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As shown in Fig 1 and Table 1 the production of infectious virus and haemagglutinin was significantly suppressed in the cultures inoculated with undiluted virus suspensions. Such cultures continuously produced low titres of infectious virus for a long period (Fig 2). A persistence of the NDV infection also occurred in secondary cultures prepared by trypsinisation of primary cultures on day 2 after virus inoculation (Fig 2).

The content of viral antigen in the cells was studied by the fluorescent antibody technique. Monolayers of calf kidney cells grown on coverslips were infected with NDV at input multiplicities of approximately 5 and 0.05 respectively. At intervals after inoculation the cells were fixed in acetone and the media assayed for haemagglutinating

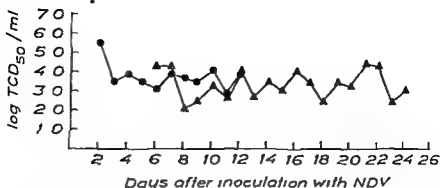


Fig 2

Yield of infectious virus in persistently infected cultures, ●—● primary cultures, ▲—▲ secondary culture, subcultivated 2 days after inoculation of undiluted virus suspension

activity. In cultures exposed to the higher multiplicity of infection the majority of cells contained fluorescent material one day after inoculation. The intensity of the fluorescence varied and in many cells there were only a few fluorescent granules (Fig 3 A). The fluorescence disappeared after further incubation (Fig 3 B) and 4 days after inoculation fluorescence could be demonstrated in only about one per cent of the cells (Fig 3 C). Fluorescence was more intense in cultures inoculated with NDV at a multiplicity of about 0.05. One day after inoculation the fluorescence occurred in only small scattered foci of cells, and on the second day almost all cells showed bright fluorescence (Fig 3 D). Further incubation of these cultures resulted in a marked cellular degeneration which was not observed in cultures inoculated with NDV at the higher multiplicity.

#### *Participation of Interferon in the Autointerference*

To examine whether the interfering activity of the virus stocks could be separated from the infectivity, samples of the stock suspensions were centrifuged at 55,000 g for 90 minutes. The supernatant was collected and the pellet resuspended in a volume of maintenance medium corresponding to the original volume. The stock suspension was further treated with trypsin since this enzyme inactivates interferon (17) but not NDV (8). The multiplication of NDV was then followed in flask cultures inoculated with the untreated virus stock, the resuspended pellet, the trypsin-treated virus or with both the supernatant and the trypsin-treated suspension. Each culture was inoculated with 1 ml of the different preparations.

As shown in Table 2 the multiplication of NDV was inhibited in cultures inoculated with untreated virus or with a mixture of trypsin-treated virus and supernatant. Higher titres of virus were produced in cultures inoculated with only the trypsin-treated virus or the resuspended pellet. These results indicated that the autointerference pheno-

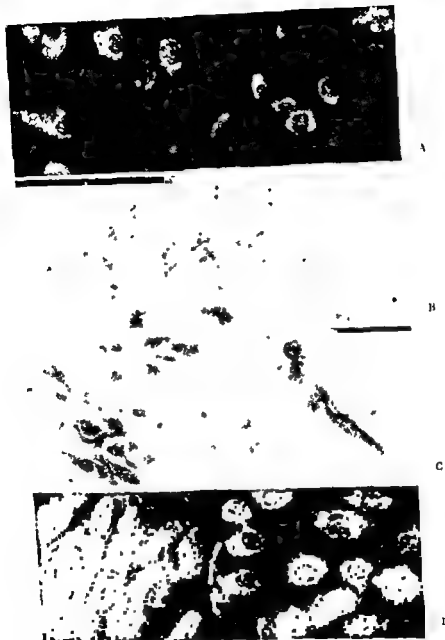


Fig 3

Micrographs of calf kidney cells infected with NDV and stained with fluorescent antibodies against this virus. Magnification  $\times 600$ . A C show cultures inoculated with NDV at a multiplicity of about 5 and incubated for 1  $\frac{1}{2}$  and 4 days respectively. D shows a culture inoculated with NDV at a multiplicity of about 0.05 and incubated for 2 days.

menon observed in cultures inoculated with undiluted virus suspensions was mainly due to the presence of an inhibitor similar to interferon. The inhibitor was therefore examined for some of the properties which characterize interferon (17, 20)

TABLE 2

*Inhibition of NDV Multiplication by a Non-Viral Factor in the Virus Suspensions*

| Inoculated suspension                | Cell degeneration per cent | Activity per ml culture fluid |          |
|--------------------------------------|----------------------------|-------------------------------|----------|
|                                      |                            | log TCID <sub>50</sub>        | HA units |
| Untreated virus                      | No                         | 5.4                           | <4       |
| Resuspended sediment*                | About 50                   | 7.4                           | 32       |
| Trypsin treated virus                | About 50                   | 7.6                           | 32       |
| Trypsin treated virus + Supernatant* | No                         | 5.5                           | <4       |

\* After centrifugation at 55 000 g for 90 minutes

Like interferon (23) the NDV inhibitor was precipitated by ammonium sulphate. A high-titered preparation was obtained by the addition of 500 g of ammonium sulphate to 1000 ml of infectious culture fluid. The sediment obtained after 18 hours at 4° C and centrifugation at 1,500 g for 30 minutes was resolved in 20 ml of medium and dialysed at 4° C against Earle's salt solution for 1 day and against maintenance medium for 1 day. Virus was removed by 2 centrifugations at 80,000 g for 2 hours. The supernatant obtained after the last centrifugation was diluted in PBS and treated with ether, trypsin, acid, DNase or RNase as described in Materials and Methods. The heat resistance of the inhibitor was tested by incubation at 70° C for 30 minutes and the sedimentation after centrifugation at 100,000 g for 2 hours. The inhibitor was further treated with NDV-antiserum for 2 hours at 37° C. The antiviral activity of the inhibitor was determined before and after the different treatments according to the technique described in Materials and Methods for assay of interferon activity.

TABLE 3

*Properties of the NDV Inhibitor*

| Treatment                               | Inhibitor titre  |                 |
|---|------------------|-----------------|
|   | Before treatment | After treatment |
| Centrifugation at 100 000 g for 2 hours | 4                | 4               |
| Incubation with NDV antiserum           | 4                | 4               |
| Dialysis against acid pH 2.0            | 4                | 4               |
| Incubation at 70° for 30 minutes        | 8                | 8               |
| Shaking with ether                      | 16               | 8               |
| Incubation with trypsin                 | 16               | <2              |
| Incubation with RNase                   | 8                | 8               |
| Incubation with DNase                   | 8                | 8               |

As shown in Table 3 dialysis at pH 2.0, incubation at 70° C. as well as treatment with nucleases or NDV-antiserum did not inactivate the inhibitor. Nor was the inhibitor sedimented by centrifugation at 100,000 g for 2 hours. Treatment with trypsin, on the other hand, clearly reduced the antiviral activity and a minor reduction in titre was obtained after shaking with ether. The inhibitor thus appeared to have several properties in common with interferon.

### The Production of Interferon

It was further studied whether interferon was produced in cell cultures where the virus multiplication was inhibited by autointerference. The experiment was performed in flask cultures infected with NDV at an input multiplicity of about 1 and 0.01 respectively. The medium was changed daily and assayed for interferon activity. Interferon in the cell phase was determined after disintegration of the cells by ultrasonic treatment for 5 minutes (11). Prior to this treatment the cells were washed three times in maintenance medium, loosened from the glass wall with a platinum wire loop and suspended in 10 ml of medium.

As shown in Table 4 interferon was produced in cultures inoculated with undiluted virus suspensions, but the maximum titre was about 4 times lower in these cultures than in the cultures inoculated with 100 fold less virus. No interferon could be demonstrated after 3 days of incubation in the former cultures. It was also observed that the release of interferon appeared to be somewhat delayed in relation to virus multiplication (for comparison see Fig. 1).

TABLE 4  
*Production of Interferon in NDV Infected Cultures*

| Days after inoculation of NDV | Interferon titre |                  |                     |                  |
|-------------------------------|------------------|------------------|---------------------|------------------|
|                               | Medium           |                  | Disintegrated cells |                  |
|                               | Dilution of NDV  |                  | Dilution of NDV     |                  |
|                               | 10 <sup>0</sup>  | 10 <sup>-1</sup> | 10 <sup>0</sup>     | 10 <sup>-1</sup> |
| 1                             | 1                | <1               | 4                   | <2               |
| 2                             | 4                | 4                | 1                   | 16               |
| 3                             | 2                | 16               | <1                  | 16               |
| 4                             | <1               | 2                | <1                  | ND               |
| 5                             | <1               | <1               | <1                  | ND               |

ND = not done

### Resistance against Challenge Infection

The antiviral resistance of NDV-infected and interferon-treated cultures

kidney

pensic

... inoculated interferon preparation, giving

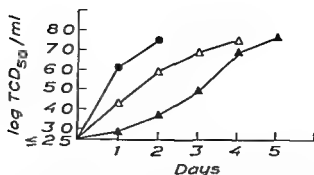


Fig 4

Resistance of calf kidney cultures to challenge infection with a bovine enterovirus after a previous infection with NDV (▲—▲) or treatment with NDV interferon (Δ—Δ) Control inoculated with challenge virus only (●—●)

a final interferon titre of 16 in the culture fluid NDV-antiserum had previously been added to the interferon preparation in order to neutralize remaining viral activity. After incubation for 3 days at 37° C these two sets of cultures and control cell cultures were inoculated with 10<sup>5</sup> TCD<sub>50</sub> of the enterovirus strain 80/61. The multiplication of the challenge virus was then followed by assaying the media for infectivity at intervals after infection.

The reproduction of the challenge virus was found to be inhibited both in cultures infected with NDV and in cultures treated with interferon (Fig 4). The inhibition of the virus multiplication was, however, greater in the NDV infected cultures, although less interferon could be demonstrated in these cultures than in cultures, supplied with exogenous interferon. Similar results were obtained when pseudorabies virus, parainfluenza virus type 1 or foot and-mouth disease virus were used for challenge.

#### Adsorption of Virus to the Cells

In several investigations dealing with viral interference the adsorption of challenge virus to the cells has not been found to be impaired (2, 10, 16, 22, 24, 26). However, a reduced ability of the cells to adsorb superinfecting virus has been established in the homologous interference between UV irradiated and active NDV in chick cells (1). Adsorption of NDV to cells showing autointerference was therefore examined. Since NDV infected cultures were more resistant against bovine enterovirus than interferon-treated cells the adsorption of the latter virus was also examined.

The experiments were performed with calf kidney cells grown in plastic petri dishes inoculated with 0.2 ml of the NDV stock suspension and incubated for 4 days at 37° C prior to the addition of the superinfecting virus. Other cultures were inoculated with 0.4 ml of NDV interferon and incubated for 2 days, some additional cultures were left uninoculated. The plates were then washed 4 times with mainte-

nance medium and inoculated with either  $10^6$  TCD<sub>50</sub> of NDV or  $10^5$  TCD<sub>50</sub> of enterovirus 80 61 in volumes of 0.2 ml. Virus was allowed to adsorb at 37° C and the plates were shaken regularly in order to spread the inoculum as a thin film on the cell sheet. After different periods of adsorption 2 ml of maintenance medium was added to the plates and the remaining viral activity of this fluid was assayed by plaque titration. The reduction of the titres was considered to be an estimate of the ability of the cells to adsorb virus.

After an adsorption period of 30 minutes approximately 10 per cent of the added amount of enterovirus was recovered in the supernates of control cultures and interferon treated cultures. On the other hand, in cultures previously infected with NDV 60 per cent of the infectivity was still unadsorbed. Similar differences were obtained at an adsorption period of 60 minutes and when the cultures were challenged with NDV (Table 5).

TABLE 5

*Adsorption of Virus to the Cells in Cultures Previously Infected with NDV or Treated with NDV Interferon*

| Challenge virus | Adsorption in minutes | Per cent residual infectivity in the fluid phase |                    |           |
|-----------------|-----------------------|--|--------------------|-----------|
|                 |                       | NDV infected                                     | Interferon treated | Untreated |
| NDV             | 30                    | 77   | 28                 | 31        |
| NDV             | 60                    | 90   | 14                 | 23        |
| Enterovirus     | 30                    | 60   | 14                 | 10        |
| Enterovirus     | 60                    | 68   | 18                 | 14        |

## DISCUSSION

The experiments reported here show that primary cultures of calf kidney cells infected with NDV produce a substance with an inhibitory effect on virus multiplication. This substance is active in all respects at (17).

It is therefore considered to be an interferon substance. Production of interferon by infection with NDV has previously been shown in MCN cells (9), mouse embryo cells (18), and in chick embryos (19).

The autointerference phenomenon which appeared in calf kidney cells

was suppressed in cultures inoculated with virus suspensions from which interferon had been removed by dilution, ultracentrifugation or treatment with trypsin.

The presence of interferon in the inocula did not prevent synthesis of viral protein in the NDV-infected cultures. After inoculation of undiluted virus suspensions and staining with fluorescent antibodies the majority of cells contained viral antigen one day after inoculation. The



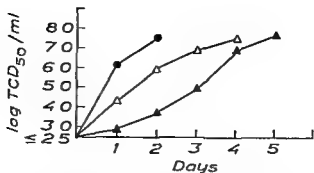


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The reproduction of the challenge virus was found to be inhibited both in cultures infected with NDV and in cultures treated with interferon (Fig 4). The inhibition of the virus multiplication was, however, greater in the NDV infected cultures, although less interferon could be demonstrated in these cultures than in cultures, supplied with exogenous interferon. Similar results were obtained when pseudorabies virus, parainfluenza virus type 1 or foot-and mouth disease virus were used for challenge.

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## DISCUSSION

The experiments reported here show that primary cultures of calf kidney cells infected with NDV produce a substance with an inhibitory effect on virus multiplication. The inhibitor appears in several respects to be similar to the interferon originally described by Isaacs et al. (17) and is therefore considered to be an interferon substance. Production of interferon by infection with NDV has previously been shown in MCN cells (9), mouse embryo cells (18), and in chick embryos (19).

The autointerference phenomenon which appeared in calf kidney cultures inoculated with undiluted suspensions of NDV seemed to be due to the presence of interferon in the inoculum. This was supported by the fact that the autointerference was suppressed in cultures inoculated with virus suspensions from which interferon had been removed by dilution ultracentrifugation.

The virus was detected in the cultures by immunofluorescence. When a suspension of cells was stained with fluorescent antibodies the majority of cells contained viral antigen one day after inoculation. The

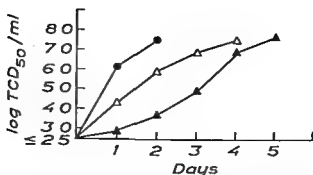


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amount of fluorescent material, however, was significantly less in these cultures than in the cultures inoculated with virus at low multiplicities. The immunofluorescence also disappeared rapidly after further incubation of the cultures inoculated with undiluted virus suspensions without any obvious degeneration of the cells. These cultures continuously produced low titres of infectious virus even after subcultivation of the cells and thus appeared to be persistently infected with NDV. A persistent infection of calf cells with NDV has previously been observed by *Dan nacher & Fedida* (6).

It has further been investigated whether virus multiplication in the persistently infected cultures is inhibited by interferon. Interferon was only detected during the first 3 days after inoculation of NDV although the titres were lower in these cultures than in cultures with more active virus multiplication. Synthesis of interferon was not demonstrated at a later stage of the persistent infection unless interferon was assayed by a more sensitive method (13) than that used in this report. On the basis of these results and in agreement with other investigations (4, 7, 9, 15, 22) it can be assumed that interferon plays a rôle for the establishment and maintenance of antiviral resistance in persistently infected cultures.

With regard to the fact that only small amounts of interferon have been demonstrated in the persistently infected cultures after the initial stage of infection (13), it must be questioned whether interferon is the only factor which maintains the carrier state. Interference in the absence of interferon has also been described. In HeLa cells chronically infected with Coxsackie virus *Crowell & Symerton* (5) found no interferon but the adsorption and penetration of the virus was decreased. However, in some other systems of persistent infections (2, 22) interferon has been demonstrated, but the adsorption of virus to the cells was not impaired. The results of the present investigation indicate that the virus adsorption is inhibited in persistently infected cultures but not in interferon-treated cultures.

The antiviral resistance was also greater in persistently infected cultures than in interferon-treated. This difference might be explained by the impaired virus adsorption in the persistently infected cultures, but it is still possible that the greater antiviral resistance is mainly due to a stronger action of endogenous interferon.

#### SUMMARY

The autointerference phenomenon which appears in calf kidney cultures infected with NDV has been associated with the presence of interferon in the inoculum. This amount of interferon does not prevent synthesis of viral protein but significantly reduces the virus yields and the cytopathic effect. The inoculation of undiluted NDV suspensions induces a persistent infection which is probably maintained not only by

production of interferon but also by a reduced ability of the cells to adsorb virus

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# ERRATA

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

67 523 1964

Table 1 second part

| Time after<br>hepatectomy | Control  | Irrad ation | Irrad ation and<br>hepatectomy | Hepatectomy |
|---------------------------|----------|-------------|--------------------------------|-------------|
| 2 days                    | 383 ± 27 | 395 ± 22    | 267 ± 28                       | 262 ± 25    |
| 14 days                   | 417 ± 48 | 424 ± 59    | 422 ± 47                       | 386 ± 69    |

*Should be*

| Time after<br>hepatectomy | Control  | Irrad ation | Irrad ation and<br>hepatectomy | Hepatectomy |
|---------------------------|----------|-------------|--------------------------------|-------------|
| 2 days                    | 383 ± 27 | 435 ± 25    | 301 ± 27                       | 262 ± 25    |
| 14 days                   | 417 ± 48 | 403 ± 48    | 415 ± 47                       | 386 ± 68    |





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## THE INCIDENCE OF LOCAL RECURRENCE AND DISTANT METASTASES IN SURGICALLY TREATED CASES OF LUNG CANCER

By

POT L S RASMUSSEN

Received 13 II 64

The demonstration of circulating tumour cells at the time of operation in patients with cancer of the lung has been a contributory cause of the present frequent use of chemotherapy as an adjuvant to surgical intervention. It is presumed that these tumour cells are particularly vulnerable to the action of cytostatics and it might be expected that these drugs given at the time of operation will prevent later development of hematogenous metastases in patients subjected to radical operation.

The frequency of circulating tumour cells in patients with cancer of the lung is reported to range from 34 per cent to 80 per cent (Klassen, Selbach & Sakurai 1962; Moore, Sandberg & Watne 1959; Pruitt, Hilberg, Morehead & Mengoli 1962), which is higher than in most other forms of cancer. However, there is no conclusive evidence of the extent to which these cells establish themselves as metastases. Engell (1959) found that patients in whom he demonstrated circulating tumour cells at the time of operation lived just as long as those in whom no tumour cells could be demonstrated. According to Moore *et al* (1959) 95 per cent-99 per cent of the cells perish in the organism.

This study was undertaken to investigate the relative incidence of local recurrence and distant metastases in a group of patients undergoing pulmonary resection for bronchogenic carcinoma. It has been of particular interest to find out how often radically operated patients die from distant metastases without local recurrence, since such metastases might be considered to originate from tumour cells spread to the blood stream immediately prior to and during operation.



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By

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I am grateful to Johannes Clemmesen MD for permission to use the files of the Danish Cancer Registry.

## MATERIAL

The material comprises a consecutive series of 227 patients who all were subjected to lung resection for bronchogenic carcinoma. In another publication (Rasmussen 1964) an account will be given of age distribution, types of resection, operative mortality, and survival rate in relation to histology and macroscopic extent of the tumour. In connection with the present study it should be mentioned that very few patients received chemotherapy, and only in cases of advanced recurrence. Apart from an isolated case X-ray treatment was only given after palliative resections and advanced recurrence.

## RESULTS

*Operative Findings*

The histological classification appears from Table 1. A macroscopical classification ad modum Salzer (1951) has been made with a few minor modifications. In accordance with Salzer the material has been arranged in various main groups, based on the local extent of the tumour, group A comprising cases where the tumour is localized to the lung parenchyma per se, Group B cases with tumour invasion of the visceral pleura, and Group C cases with invasion of the parietal pleura. Included in the latter group are all cases where tumour tissue had to be left in the bronchial stump. In addition, a division has been made based on the degree of lymphogenous spread, grade 1 comprising cases with no demonstrable glandular metastases, grade 2 cases with infiltration of interlobar and hilar glands, and grade 3 cases with infiltration of mediastinal glands. The material classified in this way appears from Table 2.

TABLE 1  
*Histological Classification*

|  |             |              |
|--|-------------|--------------|
| Squamous cell carcinoma parakeratotic      | 139 cases - | 61 per cent  |
| Squamous cell carcinoma polymorphocellular | 45 cases    | 20 per cent  |
| Undifferentiated carcinoma                 | 23 cases    | 10 per cent  |
| Adenocarcinoma                             | 20 cases    | 9 per cent   |
| Total                                      | 227 cases   | 100 per cent |

TABLE 2  
*Operative Findings in 227 Cases of Lung Cancer*

|         | Lymphatic involvement |         |         | Total |
|---------|-----------------------|---------|---------|-------|
|         | Grade 1               | Grade 2 | Grade 3 |       |
| Group A | 90                    | 40      | 30      | 160   |
| Group B | 8                     | 6       | 6       | 20    |
| Group C | 17                    | 9       | 21      | 47    |
| Total   | 115                   | 55      | 57      | 227   |

The figures read from above downwards represent increasing local extension of the tumour: Group A, tumour limited to the pulmonary parenchyma; Group B, invasion of the visceral pleura; Group C, invasion of the parietal pleura. The figures read from left to right represent increasing lymphatic involvement: Grade 1, no lymphnode involvement; Grade 2, involvement of the hilar nodes; Grade 3, involvement of the mediastinal nodes.

### Autopsy Findings

The observation period was five to eight years after which 181 patients had died. Autopsy was performed in 133 patients.

Among 62 patients who died from post operative complications within the first two months after operation, autopsy results are available in 56 cases (90 per cent). Seven of these (14 per cent) had distant metastases which were unrecognized at the time of operation.

Among 119 patients in whom death occurred later than two months after operation, autopsy results are available in 77 cases (65 per cent). In 13 of these there was no trace of the lung cancer, but in 64 there was local recurrence of the tumour and/or distant metastases. (See Table 3). Only ten patients (16 per cent of those dying from cancer of the lung) had distant metastases without local recurrence. Thus, recurrences were a dominant feature being found in 84 per cent of patients dying from cancer. In cases where local recurrence and distant metastases appeared simultaneously, the recurrence was often considerable and was usually found to be the primary cause of death.

TABLE 3

*Autopsy Findings in 64 Patients Who Died Later than Two Months after Pulmonary Resection for Lungcancer*

|   |                         |
|---|-------------------------|
| Local recurrence alone                  | 20 cases - 31 per cent  |
| Local recurrence and distant metastases | 34 cases - 53 per cent  |
| Distant metastases alone                | 10 cases - 16 per cent  |
| Total                                   | 64 cases - 100 per cent |

TABLE 4

*Autopsy Findings in Relation to the Local Extension and Lymphatic Spread of Tumour at Time of Operation*

|  | Dead of lung cancer | Recurr alone | Recurr + metast | Total recur | Metast alone | Recurr metast |
|--|---------------------|--------------|-----------------|-------------|--------------|---------------|
| Group A  | 39                  | 10           | 21              | 31          | 8            | 4/1           |
| Group B  | 10                  | 5            | 4               | 9           | 1            | 9/1           |
| Group C  | 15                  | 5            | 9               | 14          | 1            | 14/1          |
| Group A <sub>1</sub>                                   | 20                  | 3            | 12              | 17          | 5            | 3/1           |
| Group A <sub>2</sub> + A <sub>3</sub>                  | 19                  | 7            | 9               | 16          | 3            | 5/1           |
| Group A <sub>1</sub> + B <sub>2</sub> + C <sub>1</sub> | 23                  | 4            | 15              | 20          | 3            | 7/1           |

For detailed explanation of the classification see Table II and text. C<sub>1</sub> = present cases in which tumour stable lymphnode involvement to mediastinal nodes irrespective of extreme right indicates the presence (= absence of metastases) to cases with metastases alone. Metastases means distant metastases while recurrence means tumour tissue arising from the bronchial stump or lymphnodes in the hilus or mediastinum.

TABLE 5  
*Necropsy Findings in Relation to the Histological Type*

|   | Dead of<br>lung<br>cancer | Recurr<br>alone | Recurr<br>+<br>metast | Total<br>recurr | Metast<br>alone | Recurr<br>metast |
|---|---------------------------|-----------------|-----------------------|-----------------|-----------------|------------------|
| Squamous cell carcinoma para<br>keratotic group       | 32                        | 13              | 15                    | 28              | 4               | 7/1              |
| Squamous cell carcinoma poly<br>morphocell group      | 15                        | 6               | 7                     | 13              | 2               | 8/1              |
| Undifferentiated carcinoma                            | 12                        | 1               | 9                     | 10              | 2               | 5/1              |
| Adenocarcinoma  | 5                         | 0               | 3                     | 3               | 2               | 2/1              |
| Total number of resected<br>cases dead of lung cancer | 64                        | 20              | 34                    | 54              | 10              | 21               |

### *Correlation between Operative Findings and Autopsy Findings*

Such a correlation has only been made for the 119 patients who died later than two months after operation. The relative figures are given in Tables 4 and 5. The figures in the column to the extreme right express the relation between patients with local recurrences (irrespective of whether there were simultaneous metastases or not) and those with distant metastases only. As was expected, the figures show that with increasing local extent and lymphogenous infiltration a similar increase is seen in the frequency of local recurrences, particularly so in respect of the group C tumours, which all, with the exception of one case, showed local recurrence.

The figures in Table 5 show that undifferentiated carcinoma and adenocarcinoma metastasize more frequently than squamous cell carcinoma, but that also in undifferentiated carcinoma the metastases usually occur in connection with local recurrence. This phenomenon is least pronounced in the group of adenocarcinoma.

### DISCUSSION

*Bell, Gibbons & Tolstedt* (1963) performed explorative laparotomy in 30 patients with bronchogenic carcinoma and found distant metastases in six of these patients.

*Borrie* (1952), in a material of 28 patients who died postoperatively after thoracotomy for bronchogenous carcinoma, found distant metastases which had not been diagnosed at the time of operation in four patients (14 per cent).

In the present material it was found that among patients who died within the first two months after operation 14 per cent had distant metastases. It must be assumed that the vast majority of these metastases were already present at the time of operation, which is in good agreement with the observations made by *Bell et al* (1963), and by *Borrie* (1952).

The general impression received from literature is that lungcancer metastasizes frequently and extensively. In the necropsy series here referred to (*Ask Upmark 1932 Ochsner & DeBakey 1942 Fried 1948 Kahlau 1954 Callu & Payne 1955 Budinger 1958 Willis 1960 Onuigbo 1961*) which are all dominated by non operated patients the frequency of metastases is between 60 per cent and 75 per cent.

The findings derived from the present series of lung resections show a corresponding frequency of distant metastases (69 per cent) which is in good agreement with *Aylwin (1951)* who by comparing operated with non operated cases of lungcancer found distant metastases in 60 per cent and 70 per cent respectively. However if these metastases appear in connection with local recurrence the small difference between the two groups must be explained by the fact that concerning the ability to produce metastases the local recurrence must behave like a primary tumour.

The results of autopsy in the present study do in fact show that the metastases usually occur in connection with local recurrence. Distant metastases as the only tumour recurrence were only found in 16 per cent of the patients. From the observations by *Bell et al (1963)* and by *Borrie (1952)* as well as from the post mortem results of the present material of patients who died from post operative complications within the first two months after operation we know that clinically unrecognized metastases were present in a similar number of patients at the time of operation. Since metastases in none of these cases were the primary cause of death the frequency of their presence must have been the same in patients who survived the first two months. This permits of the assumption that in the group of patients where distant metastases were the only tumour manifestation the majority of these metastases must have been present prior to the operation. In other words the results of this investigation indicates that when distant metastases are found in patients following resection for lung cancer their presence can be considered either secondary to a local recurrence or be explained by the fact that they were already present but undiagnosed at the time of operation. Accordingly this study gives no support to the view that tumour cells circulating in the peripheral blood at time of operation set up unexposed metastases in patients otherwise radically treated. This is in good agreement with the observation made by *Fingell 1959* that the demonstration of tumour cells in the vascular system during operation is of no certain prognostic significance.

The present observations must be taken with the reservation that the figures are small and only two thirds of the patients underwent necropsy examination.

#### SUMMARY AND CONCLUSION

An account is given of the autopsy results in 133 patients following resection of the lung for bronchogenic carcinoma. 84 per cent of the



TABLE 5  
*Necropsy Findings in Relation to the Histological Type*

|  | Dead of<br>lung<br>cancer | Recurr<br>alone | Recurr<br>+<br>metast | Total<br>recurr | Metast<br>alone | Recurr<br>metast |
|--|---------------------------|-----------------|-----------------------|-----------------|-----------------|------------------|
| Squamous cell carcinoma para-keratotic group       | 32                        | 13              | 15                    | 28              | 4               | 7/1              |
| Squamous cell carcinoma polymorphocell group       | 15                        | 6               | 7                     | 13              | 2               | 8/1              |
| Undifferentiated carcinoma                         | 12                        | 1               | 9                     | 10              | 2               | 5/1              |
| Adenocarcinoma                                     | 5                         | 0               | 3                     | 3               | 2               | 2/1              |
| Total number of resected cases dead of lung cancer | 64                        | 20              | 34                    | 54              | 10              | 51               |

### *Correlation between Operative Findings and Autopsy Findings*

Such a correlation has only been made for the 119 patients who died later than two months after operation. The relative figures are given in Tables 4 and 5. The figures in the column to the extreme right express the relation between patients with local recurrences (irrespective of whether there were simultaneous metastases or not) and those with distant metastases only. As was expected, the figures show that with increasing local extent and lymphogenous infiltration a similar increase is seen in the frequency of local recurrences, particularly so in respect of the group C tumours, which all, with the exception of one case, showed local recurrence.

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# METHOD FOR THE IDENTIFICATION OF THE ORIGIN OF FIBRINOID AND OF OTHER INSOLUBLE PROTEINS

By

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Several chemical origins of 'fibrinoid' have been suggested: blood proteins with emphasis on fibrinogen or  $\gamma$  globulin, degenerated collagen or precipitated ground substance including the acid mucopolysaccharides (Clark, Graef & Chassy 1936, Bahrman 1937, Klemperer, Pollack & Baehr 1942, Allschuler & Angevine 1949). The most recent opinions are based on results obtained with 'fluorescent antibody' technique and favour the oldest ideas i.e., fibrinogen as the main source of fibrinoid (Gitlin, Craig & Janeway 1957), although also  $\gamma$  globulin has been demonstrated in the rheumatic noduli with the same technique (Vazquez & Dixon 1958).

The purpose of this paper is to describe a fresh chemical approach which is applicable on all insoluble proteins of obscure origin. This procedure is modified from the 'fingerprint' method of Ingram (1956, 1958) and Katz, Dreyer & Anfinsen (1959). Every protein contains an unique peptide pattern which can be visualized and identified. Ingram has demonstrated variations between similar haemoglobin proteins, which differ only by a few or even by a single amino acid in the peptide sequence. In this work the peptides were obtained by hydrolysis of the materials with trypsin. Several other proteolytic enzymes were tried also and they may be suitable for other objects.

## EXPERIMENTAL

**Hydrolysis.** The tissue samples were homogenized in water with a blender —  
genizer and the insoluble —  
c —

ples  
few

We also  
Reuma  
The rh  
M.D.R.

albumin were gifts from State Serum Institute Helsinki. The  $\gamma$  globulin and plasma

patients died from local recurrence, frequently in connection with distant metastases 16 per cent died from distant metastases without local recurrence On the basis of the literature and the results of this study it is suggested that in the latter cases the metastases were present prior to the operation in a clinically unrecognized form In other words patients resected for lung cancer does not develop metastases after the operation unless there is a local recurrence to explain their origin

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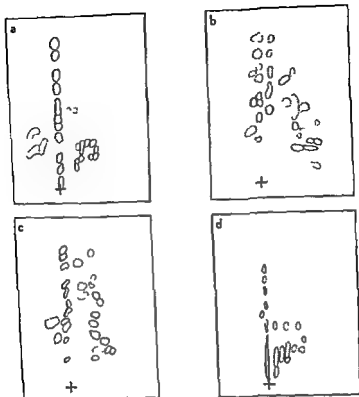


Fig 2 a-d

The peptide patterns of some trypsin digested proteins treated similarly as the rheumatic noduli: a) human fibrin clot b) human plasma  $\gamma$  globulin c) human plasma albumin d) rat tail tendon collagen

This method is suitable for the study of also other pathological substances *e.g.*, of amyloid or hyalin. Difficulties arise, when the proteins are mixtures but in principle, even then the origins could be identified by some unique peptide spots. The number of expected peptides is the same as the number of arginine plus lysine in a molecule plus one. However, in large molecules as in fibrin, the number of peptide spots is no more useful for appraisal of the proper and complete enzymatic action. The eventual large peptides yield rather faint colours with ninhydrin.

#### SUMMARY

The "fingerprint"-technique of *Ingram* was applied to the chemical identification of fibrinoid material from the rheumatic noduli. It is concluded that the trypsin digested matter resembled fibrin but not  $\gamma$  globulin, plasma albumin or gelatin.

drops of toluene were used to prevent the microbial growth. The mixture was kept at  $+37^{\circ}\text{C}$  for 24 hrs with continuous shaking and the pH was adjusted if necessary to pH 8.2–8.5 by addition of ammonium hydroxide.

The nondigested material was precipitated with 10 per cent (w/v) trichloroacetic acid (Clotten & Clotten 1962) and the supernatant cleared by centrifugation. The trichloroacetic acid was removed by repeated extractions with ether. The buffer salts are volatile and the samples could be applied on paper without any further treatment.

**Preparation of the Fingerprint Map of the Peptides** The two dimensional separation was achieved by electrophoresis in one direction and by subsequent chromatography. The electrophoresis was done by the method of Gross (1956) using Whatman No. 3 MM paper, size  $30 \times 60$  cm and pyridine acetate buffer, pH 3.4 (pyridine 17 ml, glacial acetic acid 170 ml, distilled water *ad* 5000 ml) and a voltage gradient of 40 V/cm. The system was cooled to  $+1^{\circ}\text{C}$  and the paper was insulated from the cooling blocks by 0.15 mm polythene sheets. After a run of 60 min the paper was dried at room temperature. The most basic spots travelled in these circumstances ca. 20 cm toward the cathode and the most acid ca. 5 cm toward the anode from the origin.

The chromatography was performed in conventional descending system with *n*-butanol acetic acid-water (4:1:5 by volumes) as solvent. The fastest spot travelled ca. 45 cm.

The peptides were stained by dipping the sheets into 0.2 per cent (w/v) ninhydrin solution in acetone. The colours were allowed to develop at room temperature. The 'maps' so obtained were quite reproducible.

## RESULTS

Peptide patterns of fibrinoid, gelatin and some blood proteins are shown in Fig. 1 and 2. The pattern of fibrinoid, extracted by tryptic hydrolysis from rheumatic noduli, and the respective pattern of clotted fibrin agree quite reasonably. The differences can be explained by the presence of small amounts of other proteins in the fibrinoid material.

It has been demonstrated that trypsin is useful to remove the fibrinoid from the rheumatic noduli (Ziff, Kantor, Bien & Smith 1953) although others claim that it is resistant (Glynn & Loewi 1952). This difference of opinions may be due to different methods. In our hands, the regularly stained fibrinoid material disappeared from microscopical slices after the treatment with trypsin.

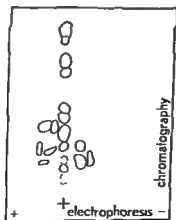


Fig. 1

The peptide pattern of trypsin digested material from rheumatic noduli

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Royal Veterinary College Stockholm

# ULTRASTRUCTURAL ALTERATIONS IN SINUSOIDAL ENDOTHELIUM OF LIVER AND BONE MARROW IN DOGS WITH EXPERIMENTAL HEPATITIS CONTAGIOSA CANIS<sup>1</sup>

By

GERT LINDBLAD & NILS BJÖRKMAN

Received 27 ii 64

The permeability of the endothelial wall has been studied by *Grotte* (1956), who found that dextran molecules with a radius of 35–45 Å could pass through the blood lymph barrier. He also postulated 'leaks' with radii of about 155 Å, being especially numerous in the liver. In a review *Renkin & Pappenheimer* (1957) dealt with the exchange process and pointed out the significance of hydrodynamic bulk flow for big molecules such as plasma protein. *Linblad et al* (1964) have shown a circulatory disturbance in connection with experimental Hepatitis contagiosa canis (Hcc). They observed an increased arteriolo venular flow ratio and, in some capillaries complete cessation of flow. The histopathological picture of Hcc has been extensively described by *Rubarth* (1947) and later by *Innes* (1949). They demonstrated lesions of varying degree in the endothelial nuclei, and intranuclear inclusion bodies. Red cells were also found in the space of Disse. Ultrastructural alterations in the sinusoidal wall have been reported in liver injuries such as allergic liver, viral liver, and bone marrow.

in the dogs and dogs with experimentally produced Hcc

## MATERIAL AND METHODS

1.

<sup>1</sup> Supported by grants from Anslag for framjande av medicinsk forskning vid Veterinarhogskolan

<sup>2</sup> The virus material used was an Hcc strain (Titre 75 TCID<sub>50</sub>/ml) obtained from R. Salenstedt, VMD National Bacteriological Laboratory, Stockholm. 4 ml of an undiluted suspension of virus were inoculated intraperitoneally in each dog.

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Fig. 1

Sinusoidal wall from normal liver of a dog. Endothelial sheet (top) surface of Disse (middle) and part of a parenchymal cell (bottom). Electron micrograph as in the following pictures  $\times 24,000$ .



Fig. 2

## RESULTS

After inoculation the dogs fell ill with varying degree of apathy, anorexia, thirst, and a temperature rise. Two of the dogs (nos. 33 and 34) were very little affected and showed no elevation of the transaminases. The results from the GOT and the OCT determinations are brought together in Table 1.

### Liver

#### Ultrastructural Observations

**Normal sinusoids.** The endothelium formed a thin cytoplasmic layer covering the parenchymal microvilli (Fig. 1). Occasionally there were



needle under thiopentone sodium anaesthesia. One dog (No 24) died 5 days after inoculation. The autopsy findings were typical of Hcc<sup>1</sup> after 32, 59 and 78 days when complete clinical recovery was observed. Samples were taken from the dog (No 22) that had shown the most severe degree of illness. Biopsy specimens were also taken from a healthy dog for controls. A further experiment was carried out on 4 dogs with experimental Hcc<sup>2</sup>. Preinoculation samples were taken for controls and frequent sampling was done until clinical recovery (see Table 1). The course of the disease was followed by frequent examinations of body temperature and the plasma activity of glutamic oxalacetic transaminase (GOT) and ornithine-carbonyl transferase (OCT) (Table 1) (cf. Lindblad & Persson 1962). From 2 of the dogs in the latter group (Nos 51 and 52) bone marrow samples from the ribs were taken at the same time, using technique described by Lindblad & Baelgren (1964). Pieces of ribs were split into 4 parts to obtain access to the bone marrows which were cut into small pieces with a razor blade. The material from the liver and bone marrow was immediately immersed in buffered osmium tetroxide solution (Mullonig 1962) and fixed for 2 hours. The specimens were embedded in Epon (Luft 1961). For identification of sinusoids, large sections 1 µ in thickness were prepared and stained with buffered toluidine blue solution (Björklund 1962). Areas free of mechanical damage were selected and the blocks were trimmed. Thin sections were prepared, stained and examined in a Siemens Elmiskop I at 60 kV.

TABLE 1  
GOT and OCT Determinations from Dogs with Experimental Hcc

| Dog no |     | Days after inoculation |      |        |       |        |       |        |      |       |      |
|--------|-----|------------------------|------|--------|-------|--------|-------|--------|------|-------|------|
|        |     | 0                      | 2    | 4      | 6     | 8      | 9     | 11     | 12   | 14    | 16   |
| 21     | GOT | 23.5                   | 27   | 40§    | 38.5§ |        | 21.5§ |        | 12   |       | 19.5 |
|        | OCT | 0                      | 3.3  | 6.1    | 5.5   |        | 2.8   |        | 1.6  |       | 1.1  |
| 22*    | GOT | 31                     | 101  | 72§    | 83§   |        | 30§   |        | 40   |       | 27   |
|        | OCT | 4.4                    | 50.5 | 20.8   | 17.3  |        | 3.4   |        | 20.5 |       | 8.0  |
| 23     | GOT | 20.5                   | 57.5 | 88§    | 134§  |        | 30§   |        | 26   |       | 18   |
|        | OCT | 1.7                    | 16.1 | 20.2   | 40.2  |        | 1.6   |        | 2.8  |       | 1.7  |
| 24     | GOT | 21.5                   | 72.5 | 510§   | Dead  |        |       |        |      |       |      |
|        | OCT | 1.6                    | 23.5 | 98.5   |       |        |       |        |      |       |      |
| 25     | GOT | 27                     | 28   | 48.5§  | 53.5§ |        | 17§   |        | 14   |       | 13   |
|        | OCT | 1.7                    | 6.6  | 13.9   | 12.7  |        | 4.4   |        | 4.5  |       | 1.6  |
| 51     | GOT | 22.5§†                 | 15§† | 21.5§† | 38§†  | 27§†   |       | 19§†   |      | 15§†  |      |
|        | OCT | 1.0                    | 3.3  | 5.6    | 11.0  | 1.6    |       | 0.6    |      | 11    |      |
| 52     | GOT | 24.5§†                 | 18§† | 49§†   | 60§†  | 72.5§† |       | 25.5§† |      | 15§†  |      |
|        | OCT | 2.6                    | 1.6  | 3.0    | 3.7   | 28.5   |       | 1.7    |      | 1.5   |      |
| 53     | GOT | 16§                    | 12§  | 21.5§  | 26§   | 17§    |       | 14.5§  |      | 16§   |      |
|        | OCT | 2.0                    | 2.5  | 1.0    | 1.9   | 4.1    |       | 0.5    |      | 3.8   |      |
| 54     | GOT | 14§                    | 16§  | 14§    | 16.5§ | 16.5§  |       | 12§    |      | 13.5§ |      |
|        | OCT | 2.1                    | 0    | 1.0    | 2.5   | 2.3    |       | 1.7    |      | 3.7   |      |

\* See the text

§ = this day biopsy specimens of the liver were taken for electron microscopical examinations

† = this day biopsy specimens of bone marrow were taken for electron microscopical examinations

1 The autopsy was carried out at the Department of Pathology, Royal Veterinary College, Stockholm.

2 The virus material used was an Hcc strain (Titre 7.5 f.c.i.d.<sub>50</sub>/ml) obtained from R. Salenstedt, VMD, National Bacteriological Laboratory, Stockholm. 4 ml of an undiluted suspension of virus were inoculated intraperitoneally in each dog.

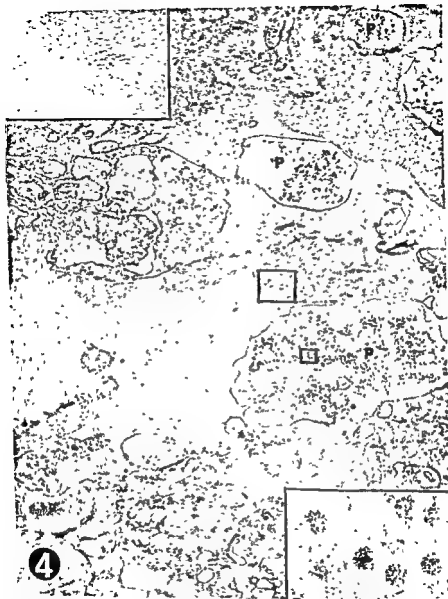


Fig. 1

Hepatic tissue showing  
the  
disappearance of  
of  
particles (dark  
fibrin clots (m  
right  $\times 18,000$



Fig 3

Hepatic sinusoidal wall with fragmentation of the endothelial cytoplasm causing open communication between the sinusoidal lumen and the space of Disse. Note the abundance of blood plasma in the lumen (top left) and the aggregations of ribosomes into dense particles in the parenchymal cell (bottom right). 4 days after inoculation  $\times 24,000$

also openings in the endothelial layer seemingly permitting free communication between the sinusoid and the space of Disse. In rare cases defects in the wall occurred which were due to mechanical damage. When Kupffer's cells occurred the sinusoidal wall was much thicker than when it consisted of ordinary endothelium.

*Sinusoids from unoculated dogs* In samples taken 2 days after ino-

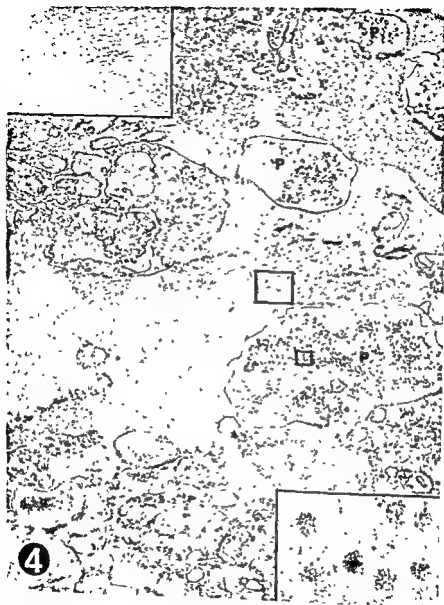


Fig. 4

Hepatic  
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right



Fig. 3

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*Sinusoids from inoculated dogs.* In samples taken 2 days after ino-



Fig 5

H pa nu o d f om the fatal case taken 4 days after nec  
 h dog l l m  
 d app a d  
 f m ro ll



Fig 5

Sinusoid from the liver of a dog 28 days after inoculation when the dog had recovered clinically and the endothelium had been restored. The wall contains a Kupffer cell (middle) and the lumen contains red cells (left). Note lipid droplets (1) in a parenchymal cell  $\times 6000$ .

culation, no differences from the normal material were observed. In samples taken from 4 to 11 days after inoculation, intact sinusoids were observed as well, but in many cases, the endothelium was damaged. The lesions varied from slight defects in the cytoplasmic sheet to complete disappearance of the endothelium with denudation of the entire surfaces of the parenchymal cells. Most alterations observed, however, were moderate although obvious. Slight alterations were seen in some endothelial cells which still showed no defects. These cells had swollen cytoplasm and swollen mitochondria, although the parenchymal mitochondria were still unchanged (Fig 2). In other cells there were swelling and fragmentation of the endothelial cytoplasm into rounded or irregular lumps offering free communication between the sinusoid and the space of Disse (Fig 3). This severe type of lesion was the most characteristic change in the affected livers. In connection with these endothelial alterations, lesions of parenchymal cells were also observed, mainly in the form of the occurrence of lipid droplets. The most severe damage consisted in a loss of endothelium and destruction of parenchymal cells. The sinusoidal lumen then contained clots of fibrin (Fig 4).

Processes of Kupffer's cells containing dense round particles of a uniform size of  $30 \text{ m}\mu$  were observed.

The above-mentioned alterations were on the whole correlated to the degree of severity of the illness as it manifested itself by clinical symp-



Fig 6

Double layer sinusoidal endothelium covering a melanocyte from bone marrow of a normal dog. Note caveolae (arrows) and pinocytotic vesicles in the endothelial cytoplasm  $\times 30,000$

toms and transaminase elevations. Thus the fatal case (no 24) presented very severe damage (Fig 4). Less severe lesions were found in dog no 22 which was the sickest among the surviving cases. In dogs nos 23 and 54 the alterations were insignificant. In specimens taken 14 days after inoculation or later there were no or insignificant alterations in the endothelium although parenchymal cells were found which had not restored their normal ultrastructure (Fig 5).

### Bone Marrow

**Normal sinusoids.** The endothelium formed sheets which were generally thicker than those found in the liver and in many cases two endothelial layers occurred (Fig 6). In most cases the endothelium was intact although defects were also seen presumably artifacts caused at the preparation.

**Sinusoids from inoculated dogs.** As in the liver the sinusoids appeared unaltered 7 days after inoculation. Endothelial lesions and subsequent restoration were observed. The course of events ran approximately parallel to that observed in the liver. As in the liver there was fragmentation of the endothelial cytoplasm (Fig 7). However a complete loss of endothelium was noted in many cases and it was therefore hazardous to determine what would actually represent a sinusoid. After 14 days the endothelium was restored and had the same appearance that in pre-inoculated cases (Fig 8).



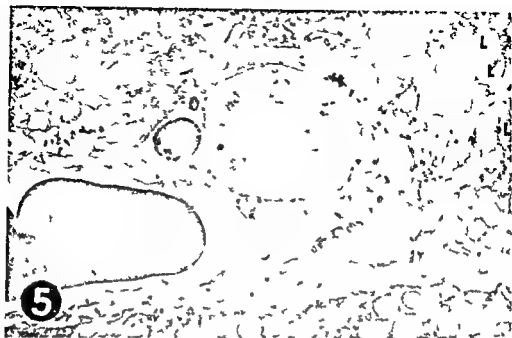


Fig 5

Sinusoid from the liver of a dog 28 days after inoculation when the dog had recovered clinically and the endothelium had been restored. The wall contains a Kupffer cell (middle) and the lumen contains red cells (left). Note lipid droplets (1) in a parenchymal cell  $\times 6000$ .

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Processes of Kupffer's cells containing dense round particles of a uniform size of  $30 \text{ m}\mu$  were observed.

The above mentioned alterations were on the whole correlated to the degree of severity of the illness as it manifested itself by clinical symp-

Alterations were not observed 2 days after inoculation, neither in the liver nor in the bone marrow. At this time the dogs from which samples were taken had a rise of temperature but no other signs or affection. After 4 days alterations were observed in most cases, and co existing clinical symptoms and increased transaminase values were observed. Restoration of the endothelium and clinical recovery were noted after 14 days.

However, the endothelial alterations vary in appearance and intact endothelium is present even in affected organs. Thus, a strict synchronism between the morphological and the clinical changes cannot unreservedly be inferred. It is worthy of note that alterations in parenchymal liver cells were seen after the sinusoids had been restored and the dogs had recovered.

The changes in the sinusoids in viral human hepatitis have been described as a narrowing or disappearance of the space of Disse (Cossel 1959) or as alterations in Kupffer's cells (Wiyat *et al* 1963). In Hcc we have found the main alterations to be swelling, fragmentation, and ballooning of the endothelial cytoplasm. A similar alteration of the microvilli has been reported in allergic injury of the liver (Steiner 1961). Although we found a fragmentation of the bone marrow endothelium the sinusoidal wall was often absent. This suggests that the injured endothelium has been swept away by the blood stream, and that hence the anchoring of the sinusoidal wall is weaker than in the liver. The particles found in processes of Kupffer's cells in the most severely affected dog were uniform in size, shape, and density, which might suggest their viral nature. The following dimensions observed under different conditions have been reported: 55-65  $m\mu$  (Tajima & Volokoski 1958; Tajima *et al* 1961), 57  $m\mu$  (Tournier *et al* 1960), 82  $m\mu$  (Davies *et al* 1961). Carmichael (1962) has reported particles of three classes: 55-60  $m\mu$ , 35-40  $m\mu$ , and 22-28  $m\mu$ . As we observed particles in only one case no definite conclusion can be drawn.

There is a normal permeability of the endothelium to particles (cf Grotte 1956) but this does not necessarily mean that there are openings in the wall (Bennett *et al* 1959) although normal opening in the sinusoidal wall have been suggested for instance by Steiner (1961). H

## SUMMARY

Biopsy specimens from the liver (10 cases) and bone marrow (2 cases) from dogs with experimentally produced Hcc were investigated with the electron microscope to study the influence of Hcc on sinusoidal endothelium. In affected dogs sinusoids showed the following alterations: Swelling of the endothelial cytoplasm, fragmentation of the cytoplasm, and loss of endothelium. In a fatal case, dense round



Fig 7

Sinusoid with fragmented endothelium from bone marrow of a dog 4 days after inoculation  $\times 19,500$

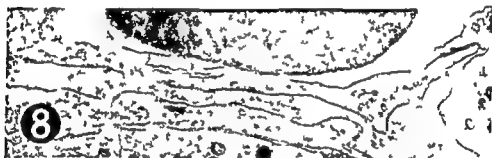


Fig 8

Double layer sinusoidal endothelium from bone marrow of a recovered dog 28 days after inoculation  $\times 18,000$

### DISCUSSION

The ultrastructure of the sinusoidal endothelium of normal dog livers presented features that have already been described in other species by e.g. Fawcett (1955) and Cosset (1959 a and b) and will not be discussed here. Corresponding endothelium from bone marrow has previously been studied with the electron microscope by Zamboni & Pease (1961). Therefore our recording of normal endothelium serves only as reference for evaluation of the pathological cases.

The methods of taking biopsy specimens involve risks of mechanical damage and in rare cases defects due to the preparation were observed in the liver material. However such artifacts could easily be distinguished from the typical alterations attributable to the illness. As regards the bone marrow artifacts were more frequent but the bone spicules supported the soft tissue and if the blocks were trimmed between adjacent spicules artifacts were mostly avoided.

Alterations were not observed 2 days after inoculation, neither in the liver nor in the bone marrow. At this time the dogs from which samples were taken had a rise of temperature but no other signs or affection. After 4 days alterations were observed in most cases, and co-existing clinical symptoms and increased transaminase values were observed. Restoration of the endothelium and clinical recovery were noted after 14 days.

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Fig 7

Sinusoid with fragmented endothelium from bone marrow of a dog 4 days after induction  $\times 12,500$



Fig 8

Double layer endothelium from bone marrow of a recovered dog 28 days after induction  $\times 18,000$

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## CHOICE OF STAINING METHOD FOR DETERMINATION OF THE PERCENTAGE OF EPITHELIUM IN THE CHICK THYROID

By

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Received 23 III 64

Young cockerels have been regarded by several authors as suitable for thyroid studies, and especially for TSH investigations. The histological studies which previously were predominant have largely been replaced by other methods. The histological evaluation of thyroid activity is still important, however, as one of the few methods which give a permanent and reproducible picture of some aspects of thyroid function.

Histological studies on the height of the acinar epithelial cells of the chick thyroid have been carried out by a number of workers (1, 5, 6, 8). The appearance of large colloid droplets in the acinar cells has been the subject of studies by *Dvoskin* (2). For assay purposes, this author compared the effect of TSH upon this reaction with the effect upon acinar cell height and thyroid weight in chicks. He found that by the colloid droplet method a dose of TSH could be demonstrated which was 1/40 of the smallest dose that could be demonstrated by the cell height method.

*Lotila & Kannas* (10) have elaborated a method for estimation of the percentage of epithelium in the thyroid by linear measurement of epithelium, colloid and stroma in histological sections. The method has been further elaborated for assay purposes in guinea pigs by *Tala* (9). It has proved especially valuable in experimental work.

Several fixing and staining methods can be used for the determination of the histological activity of the chick thyroid. The choice of method depends upon which histological reaction is to be studied. For the colloid droplet method *Dvoskin* (2) fixed his preparations in Carnoy's fluid for one hour, and stained them with his own modification of *de Robertis* (7) version of the Heidenhain azan stain. In this modification the preparations are kept in phosphotungstic acid for only one hour. With this stain, the colloid becomes intensely blue, and intracellular colloid droplets are clearly visible. The epithelium is only slightly stained but the contours of the epithelial cells are clearly visualized.

particles with uniform size of 30 m $\mu$  were found in processes of kupffer's cells. It is concluded that the damage on the endothelium increases the permeability to large particles.

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## MATERIAL AND METHODS

The material comprised 32 White Leghorn cockerels. To obtain variation in thyroid activity the animals were each given a subcutaneous injection of 0.125 USP units of TSH. This dose is above the upper limit for increased activity (4). Groups of chicks were sacrificed before and at 1, 2, 4 and 8 hours after the injection. The thyroid glands were removed and from each animal one thyroid lobe was fixed for 1 hour in Bouin's fluid, the other lobe for 1 hour in Carnoy's fluid. The lobes were embedded in paraffin and sectioned at 3  $\mu$ . The lobe fixed in Bouin's fluid was stained by Honeff's (3) modification of the Mallory azan stain, whereas the lobe fixed in Carnoy's fluid was stained by Duoskin's (2) modification of de Robertis' (7) version of the Heidenhain azan stain. The sections were projected through a microscope on a plain white surface and measured according to the method of Uotila & Kannas (10) and Tala (9) as described earlier (11). For projection an objective lens  $\times 20$  was used, the ocular was  $\times 15$ . The linear amplification was increased by the distance to the surface which was 50 cm. From each lobe 4 sections were measured, the total length measured being thus 160 cm per lobe.

The mean value and the standard error of the mean ( $e$ ) were calculated from each group of chicks with both staining methods. The standard error of the difference ( $e_d$ ) and the factor  $t$  and  $P$  values obtained by comparing the means of the different groups were also calculated.

## RESULTS AND DISCUSSION

The results are shown in Table 1 and Fig. 1. There is a good correspondence between the mean values of the results obtained by the two methods in each time group. This is seen even more clearly in Table 2. The statistical data show that the apparent differences of the mean values given by the two methods in each time group are not significant. Thus the results given by the two staining methods can be regarded as belonging to the same material, and they can be placed in the same series of observations. This has been done in Table 3, which is based on the total number of observations from both thyroid lobes of each chick. The total length measured from each animal is thus 320 cm. The table shows a good correlation between the present data and the results of previous time-response investigations (4, 11).

TABLE 2

*Statistical Relations Between the Results by the Two Staining Methods in Each Time Group*

| Time after TSH hours | $e$ | $t$   | $P$    | $t_{P=0.1}$ |
|----------------------|-----|-------|--------|-------------|
| 0                    | 3.3 | 0.39  | $>0.1$ | 1.782       |
| 1                    | 5.8 | 0.019 | $>0.1$ | 1.850       |
| 2                    | 5.2 | 1.05  | $>0.1$ | 1.812       |
| 4                    | 3.4 | 0.80  | $>0.1$ | 1.782       |
| 8                    | 4.4 | 0.50  | $>0.1$ | 1.782       |

$t_{P=0.1}$  = the value of  $t$  if  $P=0.1$  at the corresponding degree of freedom

Objections may, of course, be raised to the small number of animals examined. However, it must be noted that the  $e$  values in the different groups are of the same magnitude as those found in earlier



*Uotila & Kannas'* (10) histoquantitative method for determination of the percentage of epithelium has been used in chick studies by, for instance, *Lamberg* (4) and *Wahlberg* (11). When this method is used it is important to obtain a good contrast between colloid and epithelium. For this reason, preparations have been fixed in Bouin's fluid and stained by *Koneff's* (3) modification of the Mallory azan stain. With this stain, the colloid becomes blue and the epithelium red. Thus, this stain is well suited for the histoquantitative method.

When the *Koneff* (3) stain is used, it is not possible to obtain exact information about the intracellular colloid droplets of the chick thyroid. Hence, it was decided to test the suitability of the *Dvoskin* (2) stain for the measurement of the percentage of epithelium by *Uotila & Kannas'* (10) method, and a study was carried out in which comparable preparations of both types were used.

TABLE 1  
Percentage of Epithelium

| Time after TSH hours | Number of animals | Bouin + Koneff  |      |      | Carnoy + Dvoskin |      |      |
|----------------------|-------------------|-----------------|------|------|------------------|------|------|
|                      |                   | Number of lobes | Mean | s    | Number of lobes  | Mean | s    |
| 0                    | 7                 | 7               | 55.2 | 2.25 | 7                | 56.5 | 2.60 |
| 1                    | 5                 | 5               | 49.0 | 3.58 | 5                | 50.1 | 4.70 |
| 2                    | 6                 | 6               | 62.6 | 3.10 | 6                | 57.1 | 3.65 |
| 4                    | 7                 | 7               | 66.3 | 2.26 | 7                | 63.6 | 2.49 |
| 8                    | 7                 | 7               | 73.0 | 3.25 | 7                | 75.2 | 3.00 |

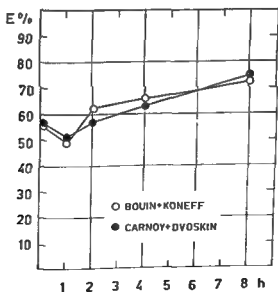


Fig. 1

Percentage of epithelium (E%) Time response relations with both methods

## MATERIAL AND METHODS

distance to the surface which was 50 cm. From each lobe 4 sections were measured the total length measured being thus 160 cm per lobe.

The mean value and the standard error of the mean ( $e$ ) were calculated from each group of chicks with both staining methods. The standard error of the difference ( $e_d$ ) and the factor  $t$  and  $P$  values obtained by comparing the means of the different groups were also calculated.

## RESULTS AND DISCUSSION

The results are shown in Table 1 and Fig. 1. There is a good correspondence between the mean values of the results obtained by the two methods in each time group. This is seen even more clearly in Table 2. The statistical data show that the apparent differences of the mean values given by the two methods in each time group are not significant. Thus the results given by the two staining methods can be regarded as belonging to the same material, and they can be placed in the same series of observations. This has been done in Table 3, which is based on the total number of observations from both thyroid lobes of each chick. The total length measured from each animal is thus 320 cm. The table shows a good correlation between the present data and the results of previous time-response investigations (4, 11).

TABLE 2  
Statistical Relations Between the Results by the Two Staining Methods in Each Time Group

| Time after<br>TSH<br>hours | $e_d$ | $t$   | $P$    | $t_{P=0.1}$ |
|----------------------------|-------|-------|--------|-------------|
| 0                          | 3.7   | 0.39  | $>0.1$ | 1.782       |
| 1                          | 5.8   | 0.019 | $>0.1$ | 1.860       |
| 2                          | 5.2   | 1.67  | $>0.1$ | 1.812       |
| 4                          | 3.4   | 0.80  | $>0.1$ | 1.782       |
| 8                          | 3.3   | 0.50  | $>0.1$ | 1.782       |

$t_{P=0.1}$  — the value of  $t$  if  $P=0.1$  at the corresponding degree of freedom.

Objections may, of course, be raised to the small number of animals examined. However, it must be noted that the  $e$  values in the different groups are of the same magnitude as those found in earlier

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|----------------------|-------------------|-----------------|------|------|------------------|------|------|
|                      |                   | Number of lobes | Mean | e    | Number of lobes  | Mean | e    |
| 0                    | 7                 | 7               | 55.2 | 2.25 | 7                | 56.5 | 2.60 |
| 1                    | 5                 | 5               | 49.0 | 3.58 | 5                | 50.1 | 4.70 |
| 2                    | 6                 | 6               | 62.6 | 3.10 | 6                | 57.1 | 3.65 |
| 4                    | 7                 | 7               | 66.3 | 2.26 | 7                | 63.6 | 2.49 |
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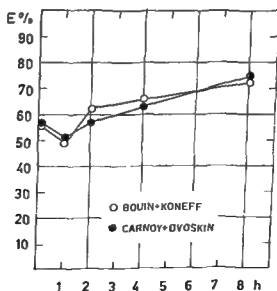


Fig. 1

Percentage of epithelium (E%) Time response relations with both methods

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investigations (11) (see Table 1) As already mentioned, pairs of slides were made with the two methods from the same animal, the same degree of stimulation for each pair being thus ensured

TABLE 3  
Percentage of Epithelium Time Response Relations Whole Material

| Time after TSH hours | Number of lobes | Mean  | $\sigma$ | $P_0^*$  | $P_a^*$            |
|----------------------|-----------------|-------|----------|----------|--------------------|
| 0                    | 14              | 75.8% | 1.59     | —        | —                  |
| 1                    | 10              | 49.5% | 8.71     | $>0.1$   | $>0.1$             |
| 2                    | 12              | 59.8% | 2.70     | $>0.1$   | $=0.01$            |
| 4                    | 14              | 64.9% | 1.61     | $<0.001$ | $>0.1$             |
| 8                    | 14              | 74.10 | 2.12     | $<0.001$ | $0.01 > P > 0.001$ |

\*  $P_0$  = P comparing the group with untreated controls

$P_a$  = P comparing the group with the one treated for the next shorter time

The results show that in investigations of the activity of the chick thyroid by *Uotila & Kannas*' (10) method, under the present test conditions, both the staining methods here employed can be used. Thus, one is free to choose between fixation with Bouin's fluid and staining by *Koneff's* (3) modification of the Mallory azan stain, and fixation with Carnoy's fluid followed by staining by *Dvoskin's* (2) modification of *de Robertis'* (7) version of the Heidenhain azan stain. Using the latter stain, one can thus choose freely between the histoquantitative method and the colloid droplet method, or use both methods on the same preparations.

#### SUMMARY

32 White Leghorn cockerels were injected with 0.125 USP units of TSH and groups were sacrificed at 0, 1, 2, 4 and 8 hours. One thyroid lobe of each animal was fixed in Bouin's fluid and stained by *Koneff's* (3) modification of the Mallory azan stain and the other lobe was fixed in Carnoy's fluid and stained with *Dvoskin's* (2) modification of *de Robertis'* (7) version of the Heidenhain azan stain. The percentage of epithelium was determined from both preparations by the method of *Uotila & Kannas* (10). The results show that the value obtained does not depend of which of the two fixing and staining methods is used. This is convenient, as earlier investigations have shown that *Dvoskin's* (2) stain is to be preferred for studies of intracellular colloid droplets.

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tion of the pituitary adrenal system when the active mobility of eosinophilic leucocytes is restricted (Osada 1961). The reticuloendothelial system eliminates eosinophilic leucocytes during glucocorticoid effect (Esselher, Jeanneret & Morand 1954). Stress which involves in addition to hormonal factors essentially neural factors leads to the accumulation of eosinopenic metabolites in the organism (Tanos, Szilasy, Varro, Eisner & Olah 1953).

The number of eosinophilic leucocytes in the lamina propria of the gastrointestinal canal obviously diminished more rapidly in the gastric mucosa under the influence of adrenocorticotrophin and glucocorticoids (Rasanen 1960) than in the mucosa of the small intestine (Sundell 1958). Tissue eosinopenia is probably caused by catabolic metabolites and its magnitude is dependent on the biologic activity of glucocorticoid (Rasanen 1962). The inhibition of the eosinopoiesis of bone marrow during glucocorticoid effect (Aschkenasy, Abulbol Piette & Layani 1956) may be a potential factor in the aetiology of tissue eosinopenia.

Eosinophilic leucocytes possibly inhibit the biologic effects of histamine (Kovacs 1950, Vercauteren 1953, 1955, Archer 1960). The superficial part of the gastric mucosa contains abundant histamine (Feldberg & Harris 1923) and mast cells (Rasanen 1958, 1960). When the mast cells degranulate, the gastric mucosa loses a great deal of histamine (Foley & Glick 1962). The histamine "in statu nascendi" which is probably liberated from mucosal mast cells during a short term stress may consume eosinophilic leucocytes on a larger scale in the superficial than in the basal part of the mucosa and then superficial tissue eosinopenia in the gastric mucosa is perhaps greater than the tissue eosinopenia in its basal part.

## METHOD

Four month old male rats of Sprague Dawley strain were used for the study. They were allowed to adapt themselves to laboratory conditions for 10 days in an animal house at a temperature of 20-22°C on water and a mixed diet ad libitum. They were divided into groups of 10 animals each for the duration of the actual experiment and subjected to the following intoxication and stress conditions:

- 1 Insulin 2 iu per rat (mean weight 177 g)
- 2 Insulin 2 iu + 0.25 mg of parathion per rat (mean weight 167 g)
- 3 Parathion 0.25 mg —
- 4 S
- 5 A
- 6 C

The

<sup>1</sup> & a 400 fold magnification (Leitz microscope plane objective and plane and wide angle eyepiece) the eosinophilic leucocytes were counted in the superficial

The Department of Pathology, Section II, University of Helsinki, Helsinki

## REGIONAL DISTRIBUTION OF TISSUE EOSINOPHILS IN THE MUCOSAL RAT STOMACH UNDER VARIOUS EXPERIMENTAL CONDITIONS, ESPECIALLY DURING SYSTEMIC STRESS<sup>1</sup>

By

TOIMI RASÄNEN

Received 14 III 64

Tissue eosinophilia in human gastric mucosa is at its most profuse in the basal part (*Hamperl 1932*); the number of eosinophilic leucocytes in gastric mucosa is c 10,000 per cum min of tissue (*Rasanen 1958*). It has been calculated that c 10 per cent of the eosinophilic leucocytes of the rat organism as a whole are located in the wall of the gastrointestinal canal and that it contains c 30 times the number of eosinophilic leucocytes in the blood (*Rytömaa 1960*).

The number of extravascular eosinophilic leucocytes is increased by foreign proteins and hypersensibilization (*Kultschitzky 1897, Biggard 1932, Speirs 1955*). Eosinophilic granulocytes proliferate in the gastric mucosa of adrenalectomised rats (*Rasanen 1961*) but not in the mucosa of the small intestine (*Sundell 1958*).

The eosinophilic leucocytes of the tissues are probably of myeloid origin travelling with blood and migrating via the wall of the capillaries (*Weinberg & Seguin 1914, Maximow 1927*). The intravascular phase—long distance transport—probably lasts 15–90 min (*White 1954, Rytömaa 1960*).

The life of eosinophilic leucocytes is c 10 days (*Osgood 1937*) which presupposes a 24-hour consumption of c  $10^5$  eosinophilic leucocytes in the rat organism. Their accumulation in the blood after resection of the ileum is indicative of their profuse elimination via the mucosa of the ileum (*Teir, Rytömaa & Cederberg 1963*). Morphologic and histo- and biochemical methods have shown that eosinophilic granulocytes are eliminated by diapedesis (*Teir, Rytömaa, Cederberg & Kiviniemi 1963*). Numerous eosinophilic leucocytes that have penetrated the intestinal epithelium have been encountered in the mucosa of the large intestine (*Maximow & Bloom 1944*). Eosinophilic leucocytes are eliminated through phagocytosis in the peritoneal cavity, especially during stimula-

<sup>1</sup> Aided with a grant from the *Sigrid Juselius Foundation* and the *Damon Runyon Memorial Fund* DRG 664 A

tion of the pituitary adrenal system when the active mobility of eosinophilic leucocytes is restricted (Osada 1961) The reticuloendothelial system eliminates eosinophilic leucocytes during glucocorticoid effect (Esselher, Jeanneret & Vorand 1954) Stress which involves in addition to hormonal factors essentially neural factors leads to the accumulation of eosinopenic metabolites in the organism (Tanos, Szilasy, Varro, Eisner & Olah 1953)

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and add

## METHOD

7

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The insulin (Insulin Medica) and ACTH (Cortrophin Organon) were injected in aqueous solution intramuscularly and the ethanol solution of commercial parathion intra abdominally

The rats were killed by decapitation at the times shown in Table 1 The abdominal cavity was opened immediately the stomach was removed and opened its contents emptied carefully and spread on a paperboard strip Fixation was performed in Bouin's solution Paraffin sections 4  $\mu$  in thickness were cut as perpendicularly as possible to the mucosa and stained with haemalum eosin after removal of the paraffin

Using a 400 fold magnification (Leitz microscope plan objective and plane and wide angle eyepiece) the eosinophilic leucocytes were counted in the superficial



and basal layer of the corpus mucosa over a length of 3.5 mm from sites corresponding to one another. The means and their standard error are given in the results. The significance of the differences of the means was obtained from Fisher and Yates's "Statistical Tables for Biological, Agricultural and Medical Research" (1938).

## RESULTS

The eosinophilic leucocytes counts per sq mm are shown in Table 1. The number of tissue eosinophilic leucocytes in the superficial part of body mucosa decreased after a short period of insulin- or parathion-induced shock. The same phenomenon was noted during swimming stress. Superficial eosinophilia appeared to decrease less under the influence of adrenocorticotrophin and the difference was not statistically significant. No statistically demonstrable change was established in the eosinophil count of the basal part.

TABLE 1

*The Distribution of Tissue Eosinophils in the Superficial and Basal Part of Rat Gastric Mucosa during the Short-Time Effect of Shock, ACTH and Stress*

| Treatment           | Time in minutes | No of cases | Superficial eosinophilia | P    | Basal eosinophilia | P < |
|---------------------|-----------------|-------------|--------------------------|------|--------------------|-----|
| Insulin 2 i.u.      | 300             | 10          | 40 $\pm$ 10.7            | 0.01 | 673 $\pm$ 86       |     |
| Insulin 2 i.u.      |                 |             |                          |      |                    |     |
| + parathion 0.25 mg | 15-30           | 10          | 26 $\pm$ 13              | 0.01 | 624 $\pm$ 90       |     |
| Parathion 0.125 mg  | 15-60           | 10          | 79 $\pm$ 28              | 0.05 | 637 $\pm$ 87       |     |
| ACTH 15 i.u.        | 300             | 10          | 112 $\pm$ 22             |      | 665 $\pm$ 63       |     |
| Swimming stress     | 140-230         | 10          | 62 $\pm$ 9               | 0.01 | 620 $\pm$ 69       |     |
| Controls            |                 | 10          | 169 $\pm$ 27             |      | 891 $\pm$ 82       |     |

Fairly numerous leucocytes with annular nuclei and eosinophils that stained poorly in the peripheral protoplasm in the superficial lamina propria of the mucosa were established in the rats given parathion. They were obviously eosinophilic leucocytes which had become degranulated. These changes were not demonstrable in the eosinophilic leucocytes in the basal part of the mucosa, whose granulation was profuse. Eosinophilic leucocyte granulation in the superficial part of the gastric mucosa of the control group rats was normal.

The gastric mucosa of the rats given adrenocorticotrophin or only insulin and subjected to the swimming stress displayed a decrease in eosinophilic leucocyte granulation on moving towards the surface of the mucosa, although degranulated leucocytes with annular nuclei were scantier in these cases than in connection with parathion intoxication when the shock was rapid and severe.

Eosinophilic leucocytes were not encountered in the lumina of the glandular tubules in the gastric mucosa, nor were they found to migrate via the superficial epithelium into the gastric lumen. They were located solely in the lamina propria and muscularis mucosae and here and there in the submucosa.

## DISCUSSION

Eosinophilic leucocytes appear to undergo a rapid degranulation process in the superficial part of the gastric mucosa during acute shock and stress without any detectable signs of cell destruction in the nucleus of the cell. Eosinophilic leucocytes are obviously destroyed in the basal part of gastric mucosa during glucocorticoid effect, parallel with degranulation there is nuclear fragmentation (Rasanen 1962) such as occurs in the blood (Padawer & Gordon 1952).

The superficial part of the lamina propria of gastric mucosa contains a profusion of mast cells which degranulate rapidly during stress. Their number in an earlier study was as follows (Rasanen 1963)

|         | Insulin | Insulin +<br>parathion | Parathion | Swimming<br>stress | ACTH | Controls |
|---------|---------|------------------------|-----------|--------------------|------|----------|
| Medians | 291     | 390                    | 874       | 594                | 511  | 1042     |
| P <     | 0.01    | 0.001                  |           | 0.02               | 0.03 |          |

Acetylcholine liberated by an insulin induced vagal stimulation, the disintegration of which is inhibited by parathion, possibly functions as a histamine liberator in gastric mucosa. During gastric stimulation histamine appears in gastric juice and increases in the portal blood (Silen & Eiseman 1961). Topical histamine liberation in the superficial part of the lamina propria of gastric mucosa may accelerate the degranulation of eosinophilic leucocytes which probably occurs gradually, while they first undergo vacuolization.

After vagotomy and atropine which inhibit the vagal secretory effect on gastric mucosa the quantity of eosinophil granulocytes increases in rat gastric mucosa (Teir, Wegelius, Sundell, Pajvarinne & Huusi 1955).

The fairly slight loss of eosinophilic granulocytes of tissue in the superficial part of the mucosa after a large single dose of adrenocorticotrophin may be due to the hormonal reaction in the gastric mucosa producing changes that are slower than those caused by neural stimulation (Rasanen 1963). Similar eosinopenia has been induced in human gastric mucosa within 8 hours of a single injection of ACTH (Suurala, Rasanen & Sundberg 1959). It occurred in a phase in which the mast cells were probably already in a regranulation phase.

During stress histidine decarboxylase activity increases in tissues (Schayer 1960) but the histamine detoxicating histaminase also increases especially in intestinal mucosa when the pituitary-adrenal system is stimulated (Kahlson 1956). The histaminase-histamine system undergoes coupled oxidation, presumably by the action of hydrogen peroxide and catalase which serves as a peroxidase. Peroxidase is found in eosinophilic leucocytes (Vercauteren 1955, Rylomaa 1960, Archer & Broome 1963) and the peroxidase content of the tissues depends on their eosinophilic leucocyte content (Rylomaa & Teir 1961). The great number of eosinophils in intestinal mucosa and their peroxidase content may cause profuse acetylation of histamine in it.

and basal layer of the corpus mucosa over a length of 3.5 mm from sites corresponding to one another. The means and their standard error are given in the results. The significance of the differences of the means was obtained from Fisher and Yates's 'Statistical Tables for Biological Agricultural and Medical Research' (1938).

## RESULTS

The eosinophilic leucocytes counts per sq mm are shown in Table 1. The number of tissue eosinophilic leucocytes in the superficial part of body mucosa decreased after a short period of insulin- or parathion-induced shock. The same phenomenon was noted during swimming stress. Superficial eosinophilia appeared to decrease less under the influence of adrenocorticotrophin and the difference was not statistically significant. No statistically demonstrable change was established in the eosinophil count of the basal part.

TABLE 1

*The Distribution of Tissue Eosinophils in the Superficial and Basal Part of Rat Gastric Mucosa during the Short Time Effect of Shock, ACTH and Stress*

| Treatment           | Time in minutes | No of cases | Superficial eosinophilia | P    | Basal eosinophilia | P < |
|---------------------|-----------------|-------------|--------------------------|------|--------------------|-----|
| Insulin 2 i.u.      | 300             | 10          | 40 $\pm$ 10.7            | 0.01 | 673 $\pm$ 86       |     |
| Insulin 2 i.u.      |                 |             |                          |      |                    |     |
| + parathion 0.25 mg | 15-30           | 10          | 26 $\pm$ 13              | 0.01 | 624 $\pm$ 90       |     |
| Parathion 0.125 mg  | 15-60           | 10          | 79 $\pm$ 28              | 0.05 | 637 $\pm$ 87       |     |
| ACTH 15 i.u.        | 300             | 10          | 112 $\pm$ 22             |      | 665 $\pm$ 63       |     |
| Swimming stress     | 140-230         | 10          | 62 $\pm$ 9               | 0.01 | 620 $\pm$ 69       |     |
| Controls            |                 | 10          | 169 $\pm$ 27             |      | 891 $\pm$ 82       |     |

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(Anrep, Ayadi, Barsoum, Smith & Talaat 1944). The rat organism is hardly capable of histamine acetylation after enterectomy (Millican 1953).

Eosinophilic leucocytes appear to have affinity for phagocytosis of mast cell granules (Welsh & Geer 1959) and when they proliferate in tissue containing mast cells the histamine content decreases (Riley 1956). In tissue extract histamine binds with arginine (Archer 1958) which has been isolated also from eosinophilic leucocytes and possibly has an antihistaminic effect (Vercauteren 1953, 1955). Eosinophilic leucocytes probably bind histamine (Kovacs 1950, Vaughn 1953) and their suspension has an antihistaminic effect (Vercauteren 1955, Archer 1960 a, b, Broome & Archer 1962, Esch & Taubert 1963).

Degranulation of gastric mucosal mast cells is obviously accompanied by histamine liberation (Foley & Glick 1962) and stimulation of parenchymal cells. Eosinophilic leucocytes possibly detoxicate the excess histamine in the lamina propria of the stomach which is reversed during secretion. This process may be significant as a consumer of eosinophilic leucocytes. Migration of eosinophilic leucocytes via the epithelium of gastric mucosa like that observed in the mucosa of the ileum (Teir *et al* 1963) was not established in the present work.

As regards the function of the gastric parenchyma, the profusion of eosinophilic leucocytes in the gastric mucosa of patients with gastric ulcer and gastric cancer (Rasanen 1958) may have an inhibitory effect on gastric secretion by binding and destroying endogenous histamine.

#### SUMMARY

It has actually been observed recently that the elimination of eosinophilic granulocytes appears to take place largely via the gastrointestinal canal. Their regional distribution in the gastric mucosa is of interest in this respect. Therefore, the distribution was studied by counting the number of eosinophilic leucocytes separately in the basal and superficial part of the gastric body mucosa of the rat after insulin shock, parathion intoxication, adrenocorticotrophin injection and swimming stress.

The eosinophilic leucocyte count decreased distinctly in the superficial part of the lamina propria during insulin shock, parathion intoxication and stress. The reduction in number was due to their degranulation. Superficial tissue eosinopenia was scanty after the administration of adrenocorticotrophin.

No demonstrable change in the number of eosinophilic leucocytes occurred during this time in the basal part of the mucosa nor were forms suggestive of their degranulation encountered there.

The potential rôle of eosinophilic leucocytes in the elimination of topical histaminemia while eosinophilic granulocytes participate in both the binding of histamine and its enzymatic degradation is discussed.

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## STUDIES ON MONONUCLEAR CELLS OBTAINED FROM SYNOVIAL FLUID OF PATIENTS WITH DIFFERENT TYPES OF ARTHRITIS. CYTOTOXIC EFFECT ON TISSUE-CULTURED HUMAN FIBROBLASTS

By

HELGE HEDBERG and BENGT KÄLLEN

Received 10 III 64

For several years, many authors have speculated whether immunological mechanisms are involved in the pathogenesis of rheumatoid arthritis. Rheumatoid arthritis is also generally included in synopses of so called autoimmune diseases (see, for example, the reviews of Dameshek *et al* 1961, Milgrom & Witebsky 1962, Waksman 1962, Mackay & Burnet 1963). Experimental counterpart of rheumatoid arthritis is dubious. Thus one of the fundamental postulates by Witebsky (1957) and Milgrom & Witebsky (1962), *i.e.* that of a passive transfer of the experimental disease, is not fulfilled.

Many studies have demonstrated that the various experimental autoimmune conditions are mediated by cell-bound immunologic mechanisms of the so called delayed hypersensitivity or tuberculin type. Transfer experiments in experimental allergic encephalomyelitis (Paterson 1960, Stone 1961, Åström & Waksman 1962) and in experimental allergic thyroiditis (Felix-Davies & Waksman 1961) have shown that these diseases can be transferred with suspensions of lymph node cells, whereas passive transfer with serum has failed. Waksman & Wennersten (1963) have reported a successful transfer of experimental adjuvant arthritis in rats by means of lymph node cells, and Hess *et al* (1962) transferred autoimmune nephrosis in rats with lymph node cells.

Usually it is not possible to carry out transfer experiments for the purpose of directly studying whether lymphoid cells play any rôle in the pathogenesis of human diseases. During the last few years, some different *in vitro* systems have been developed which would make such studies feasible. In one of these systems, lymphocytes from the patient are combined with living "target" cells kept in tissue culture. In animal experimental autoallergic diseases, such models have worked satis-



factorily. Thus *Koprowski & Fernandes* (1962) showed that lymph node cells from animals with experimental allergic encephalomyelitis accumulate around glia cells, kept *in vitro* (contactual agglutination), and cause glia cell lysis. *Berg & Kallen* (1963) described the same phenomenon using white blood cells, isolated from peripheral blood. Studying experimental allergic thyroiditis in rabbits (*Rose et al* 1963) and rats (*Bjorklund* 1964), a similar cytotoxic effect of lymphoid cells on cultured thyroid cells has been demonstrated. A parallel can also be drawn to the experimental homograft situation where host lymph cells *in vitro* can be shown to attack and destroy donor cells (*Govaerts* 1960, *Rosenau & Moon* 1961, and others).

This method was adopted by *Berg & Kallen* (1964) in a study on multiple sclerosis. They could demonstrate that mononuclear cells isolated from the peripheral blood of many such patients selectively attack cultured glia cells and exert a cytotoxic effect. *Perlmann & Broberger* (1963) described a cytolytic activity of lymphoid cells from patients with ulcerative colitis when added to human foetal colon cells *in vitro*.

In the present paper, a similar study has been undertaken using lymphoid cells taken from the synovial fluid of patients with various forms of arthritis. *Braunsteiner et al* (1963) described a slight, cytotoxic effect of lymph node cells from five rheumatoid arthritis patients on trypsinized cultured human amnion cells. Contactual agglutination was also noted. Control lymph node cells lacked this activity. However, these authors did not study other types of arthritis.

## CLINICAL MATERIAL

The clinical material comprised 38 patients listed in Table 1: all patients except four were or had been admitted to the Department of Rheumatology, University Hospital Lund. The patients were adult if not otherwise stated. RA patients with subcutaneous nodules and non-rheumatoid cases (see Table 1) were specifically chosen, which explains the high proportion of such cases. The others were randomly selected among patients with effusion of the knee joint(s). Only effusions amounting to at least 20 ml were examined. The majority of the patients studied—rheumatoid as well as non-rheumatoid—had shown effusion of the knee joint for several years, repeatedly treated with aspirations and with injections of hydrocortisone. No patient had received such injections during the three weeks preceding examination.

### *Rheumatoid Arthritis (RA) and Systemic lupus erythematosus (SLE)*

In the classification of RA the criteria proposed by *Ropes et al* (1958) were mainly followed. All those labelled 'classical RA' showed a positive sensitized sheep cell (SSC) test and the majority had been consistently positive in this test. Included as a classical RA was one juvenile RA. Four patients with a negative SSC test were labelled 'definite RA'. Three of these patients had been consistently negative in the SSC test; the fourth, who had been afflicted with the disease for six months, had been tested only once.

One patient with a typical *Felty's syndrome* was also included.

Three patients with systemic lupus erythematosus, called SLE below—or suspected SLF—were studied. One showed positive LE cell tests, a low complement level of serum, and slight leucopenia. The second had a doubtful rash, intermittent fever, carditis, severe anaemia, nephropathy, and a false positive test for syphilis. The

third had intermittent fever nephropathy and a false positive test for syphilis. In the two last mentioned cases repeated LE cell tests had been negative. All three showed arthritis of the rheumatoid type.

The cases of SLE were considered clinically active. The RA cases showed a varying degree of activity.

#### *Von Rheumatoid Arthritis (See Table 1)*

*Osteoarthritis* three patients

*Chronic uro polyarthritis* (definition Olhagen 1960) three patients. Two had sacro ilitis and one had active prostatic vesiculitis.

*Ankylosing spondylitis* five patients. Two of these presenting the juvenile forms. All had erosions and/or sclerosis of the sacro iliac joints. X ray changes indicating spondylitis were present in four of these patients. One juvenile case presented no X ray changes of the spine. Clinically all showed signs of involvement of the spine.

*Atypical polyarthritis* two patients with main involvement of the big joints. Both had consistently negative SSC tests.

joints in hands and feet, the terminal joints not being affected.

The SSC test as well as the Latex agglutination test were negative in all non rheumatoid patients. The latter test—when performed—was negative also in synovial fluid from these patients.

The majority of the non rheumatoid cases was considered clinically active.

## METHODS

Synovial fluid was drawn from the patient and treated with hyaluronidase (120 viscosity reducing units/ml 37° C 15 mins) and heparinized. A separation of the white blood cells into mononuclears and granulocytes was made according to the method described by Walker & Palmer (1962) for whole blood. The synovial fluid

cell suspension was made in Parker 199 to give a final cell concentration of

1 x 10<sup>6</sup>

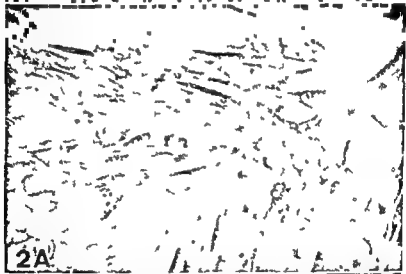
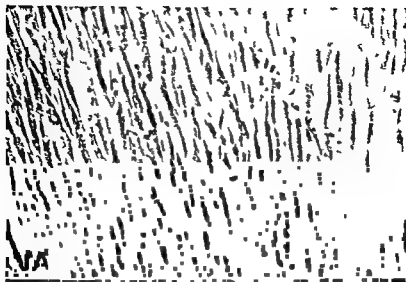
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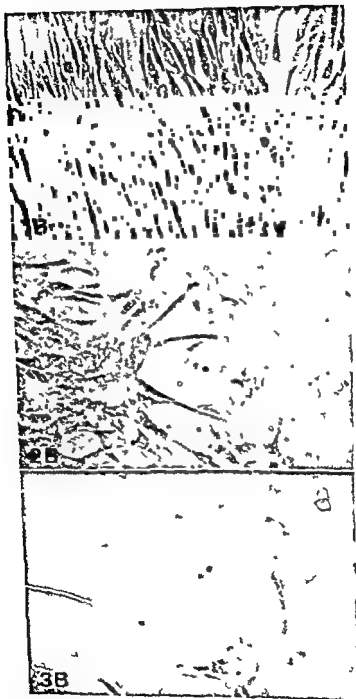
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mononuclears separated as described above were added (volume 1 ml) to the fibroblast cultures which previously were rinsed with pre warmed Parker 199. Mononuclears were left in contact with the cultures for 15 min. then removed. The cell growth was good. The cell count was calculated. The cell count was





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1. The total number of variables is 22

2. The total number of variables is 22

3. The total number of variables is 22

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2A

In Table 1, some data are given of the cases studied together with the outcome of the cytotoxicity tests. Repeated tests with mononuclears taken at varying intervals (up to 4 months) from six different patients gave the following results: in two patients tests were positive on both occasions, in three negative on both occasions, in one patients (diagnosis psoriatic arthropathy) one test was positive, the second test, 4 months later, being negative.

Positive cytotoxicity tests were found as is apparent from Table 1, in patients with rheumatoid arthritis, SLE, and psoriatic arthropathy.

In order to demonstrate the possible effect of added serum on the cytotoxicity tests, some preliminary data are given in Table 2. As is evident from this table, three different patterns of serum effect were observed.

TABLE 2  
The Cytotoxic Effect of Mononuclear Cells without Addition of and in the Presence of Fresh Serum \*

| Diagnosis                | No. of patients | Cytotoxic effect of mononuclear cells only | Cytotoxic effect of mononuclear cells with |                   |
|--------------------------|-----------------|--|--|-------------------|
|                          |                 |  | Patient serum                              | Blood donor serum |
| A                        |                 |  |  |                   |
| Non rheumatoid arthritis | 3               | 0  | 0  |                   |
| Definite RA              | 2               | 0  | 0  | 0                 |
| Class RA                 | 4               | 0  | 0  | 0§                |
| B                        |                 |  |  |                   |
| Felty's syndrome         | 1               | 0  | ++   |                   |
| Definite RA              | 1               | 0  | ++   | 0                 |
| Class RA                 | 1               | 0  | +  | 0                 |
| C                        |                 |  |  |                   |
| SLE                      | 1†              | ++   | 0  | 0                 |
| Class RA                 | 3               | +  | 0  | 0                 |
|                          | 1               | ++   | 0  |                   |

\* Within each group of patients tests were performed with 25 and with 50 per cent of serum.

§ Mononuclear cells from two patients not tested in combination with blood donor serum.

† SSC test was positive (see also Table 1).

In Group A, 11 cases are shown in which mononuclears without serum exerted no cytotoxic effect. Addition of the patient's own serum or blood donor serum did not influence the outcome of the tests.

In Group B, 3 cases are shown without cytotoxic effect of mononuclears only. After addition of the patient's fresh serum, a "++" effect was obtained, while the addition of blood donor serum in two of the cases did not provoke cytotoxicity. The patient's serum without mononuclears had no cytotoxic effect. Each serum was combined with non cytotoxic mononuclears from non rheumatoid patients. No cytotoxicity appeared.

but did not seem to influence the outcome of the test, neither did variations in cell counts and percentage of granulocytes

The tests for rheumatoid factor and the estimation of synovial complement (C) activity were performed as previously described by Hedberg (1963) Values of the synovial C activity between + 0.20 and -0.20 were considered normal, values below -0.20 were considered suppressed

## RESULTS

The cytotoxicity tests were scored "0", "+", and "++", as shown and described in Figures 1-3 Tests scored "+" and "++" were considered positive

TABLE 1

*The Cytotoxic Effect of Mononuclear Cells on Human Fibroblasts (without Addition of Serum) in Different Types of Arthritis*  
SSC = sensitized sheep cell

| Diagnosis                  | No of patients | Post the SSC test | Cytotoxic effect* |   |    |
|----------------------------|----------------|-------------------|-------------------|---|----|
|                            |                |                   | 0                 | + | ++ |
| SLE                        | 1              | 0                 |                   |   | 1  |
| SIE                        | 2              | 2                 |                   |   | 2  |
| Total                      | 3              | 2                 |                   | 3 |    |
| Feltz's syndrome           | 1              | 1                 | 1                 |   |    |
| Classical RA               |                |                   |                   |   |    |
| A With subcutan nodules    | 4              | 4                 | 1                 |   | 3  |
| B Without subcut nodules   | 11             | 11                | 7                 | 3 | 1  |
| Total                      | 15             | 15                | 8                 | 7 |    |
| Definite RA                | 4              | 0                 | 4                 |   |    |
| Non rheumatoid arthritis § |                |                   |                   |   |    |
| A Psoriatic arthropathy    | 2              | 0                 |                   |   | 2  |
| B Other †                  | 13             | 0                 | 13                |   |    |

\* See Figs 1-3 and text

§ This term was used for SSC negative cases of arthritis which were not labelled SLE or RA

† Osteoarthritis 3 patients, chronic uro polyarthritis 3 patients, ankylosing spondylitis 5 patients, atypical polyarthritis 2 patients

### Figs 1-2 and 3

Fig 1 "0" A Fibroblast culture before addition of SSC field 20 hours later Phase contrast

Fig 2 "+" A Fibroblast culture before addition of SSC field 20 hours later Phase contrast

Fig 3 Outcome of cytotoxicity test A Fibroblast culture before addition of mononuclears B Same microscopic field 18 hours later Phase contrast—Nearly complete dissolution of fibroblast mat Note aggregation of lymphocytes around residual fibroblast "contactual agglutination"

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| Definite RA              | 2              | 0  |  | 0                 |
| Class RA                 | 4              | 0  | 0  | 0§                |
| B                        |                |  |  |                   |
| Felty's syndrome         | 1              | 0  | ++   |                   |
| Definite RA              | 1              | 0  | ++   | 0                 |
| Class RA                 | 1              | 0  | +  | 0                 |
| C                        |                |  |  |                   |
| SLE                      | 1†             | ++   | 0  | 0                 |
| Class RA                 | 3              | +  | 0  | 0                 |
|                          | 1              | ++   |  |                   |

\* Within each group of patients tests were performed with 25 and with 50 per cent of serum.

§ Mononuclear cells from two patients not tested in combination with blood donor serum.

† SSC test was positive (see also Table 1).

In Group A 9 cases are shown in which mononuclears without serum exerted no cytotoxic effect. Addition of the patient's own serum or blood donor serum did not influence the outcome of the tests.

In Group B 3 cases are shown without cytotoxic effect of mononuclears only. After addition of the patient's fresh serum a ++ effect was obtained while the addition of blood donor serum in two of the cases did not provoke cytotoxicity. The patient's serum without mononuclears had no cytotoxic effect. Each serum was combined with non cytotoxic mononuclears from non rheumatoid patients. No cytotoxicity appeared.



but did not seem to influence the outcome of the test, neither did variations in cell counts and percentage of granulocytes

The tests for rheumatoid factor and the estimation of synovial complement (C) activity were performed as previously described by Hedberg (1963). Values of the synovial C activity between +0.20 and -0.20 were considered normal, values below -0.20 were considered suppressed

## RESULTS

The cytotoxicity tests were scored "0", "+", and "++", as shown and described in Figures 1-3. Tests scored "+" and "++" were considered positive.

TABLE 1

*The Cytotoxic Effect of Mononuclear Cells on Human Fibroblasts (without Addition of Serum) in Different Types of Arthritis*  
SSC = sensitized sheep cell

| Diagnosis                  | No of patients | Positive SSC test | Cytotoxic effect <sup>a)</sup> |   |    |
|----------------------------|----------------|-------------------|--------------------------------|---|----|
|                            |                |                   | 0                              | + | ++ |
| SLF                        | 1              | 0                 |                                |   | 1  |
| SLF                        | 2              | 2                 |                                |   | 2  |
| Total                      | 3              | 2                 |                                |   | 3  |
| Icterus syndrome           | 1              | 1                 | 1                              |   |    |
| Classical RA               |                |                   |                                |   |    |
| A With subcutan nodules    | 4              | 4                 | 1                              |   | 3  |
| B Without subcut nodules   | 11             | 11                | 7                              | 3 | 1  |
| Total                      | 15             | 15                | 8                              |   | 7  |
| Definite RA                | 4              | 0                 | 4                              |   |    |
| Non rheumatoid arthritis § |                |                   |                                |   |    |
| A Psoriatic arthropathy    | 2              | 0                 |                                |   | 2  |
| B Other †                  | 13             | 0                 | 13                             |   |    |

<sup>a</sup> See Figs 1, 3 and text

§ This term was used for SSC negative cases of arthritis which were not labelled SLF or RA

† Osteoarthritis 3 patients, chronic uro polyarthritis 3 patients, ankylosing spondylitis 5 patients, atypical polyarthritis 2 patients

Figs 1, 2 and 3

Fig 1 0 A Fibroblast culture before addition of sheep cell 20 hours later Phase contrast

Fig 2 + A Fibroblast culture before addition of sheep cell 20 hours later Phase contrast—Slow and hours beginning

Fig 3 Outcome of cytotoxicity test scored ++ = A fibroblast culture before addition of mononuclears B Same microscopic field 18 hours later Phase contrast—Nearly complete dissolution of fibroblast mat. Note aggregation of lymphocytes around residual fibroblast, contactual agglutination

In Table 1, some data are given of the cases studied together with the outcome of the cytotoxicity tests. Repeated tests with mononuclears taken at varying intervals (up to 4 months) from six different patients gave the following results: in two patients tests were positive on both occasions, in three negative on both occasions, in one patients (diagnosis psoriatic arthropathy) one test was positive, the second test, 4 months later, being negative.

Positive cytotoxicity tests were found, as is apparent from Table 1, in patients with rheumatoid arthritis, SLE, and psoriatic arthropathy.

In order to demonstrate the possible effect of added serum on the cytotoxicity tests, some preliminary data are given in Table 2. As is evident from this table, three different patterns of serum effect were observed.

TABLE 2

*The Cytotoxic Effect of Mononuclear Cells without Addition of and in the Presence of Fresh Serum\**

| Diagnosis                | No of patients | Cytotoxic effect of mononuclear cells only | Cytotoxic effect of mononuclear cells with |                   |
|--------------------------|----------------|--|--|-------------------|
|                          |                |  | Patient serum                              | Blood donor serum |
| <b>A</b>                 |                |  |  |                   |
| Non rheumatoid arthritis | 3              | 0  | 0  |                   |
| Definite RA              | 2              | 0  | 0  | 0                 |
| Class RA                 | 4              | 0  | 0  | 0½                |
| <b>B</b>                 |                |  |  |                   |
| Felty's syndrome         | 1              | 0  | ++   |                   |
| Definite RA              | 1              | 0  | ++   | 0                 |
| Class RA                 | 1              | 0  | +  | 0                 |
| <b>C</b>                 |                |  |  |                   |
| SLE                      | 1†             | ++   | 0  | 0                 |
| Class RA                 | 3              | +  | 0  | 0                 |
|                          | 1              | ++   | 0  |                   |

\* Within each group of patients tests were performed with 20 and with 50 per cent of serum.

½ Mononuclear cells from two patients not tested in combination with blood donor serum.

† SCL test was positive (see also Table 1).

In Group A, 9 cases are shown in which mononuclears without serum exerted no cytotoxic effect. Addition of the patient's own serum or blood donor serum did not influence the outcome of the tests.

In Group B, 3 cases are shown without cytotoxic effect of mononuclears only. After addition of the patient's fresh serum, a "++" effect was obtained while the addition of blood donor serum in two of the cases did not provoke cytotoxicity. The patient's serum without mononuclears had no cytotoxic effect. Each serum was combined with non-cytotoxic mononuclears from non rheumatoid patients. No cytotoxicity appeared.

In Group C finally 5 cases are shown in which the mononuclears produced a cytotoxic effect which was extinguished by addition either of the patient's own fresh serum or of blood donor serum—fresh or heat inactivated.

In one case of SII and one of class RA the cytotoxic effect of mononuclears was not at all or only partially neutralized by the patient's serum inactivated at 56° C for 30 mins. In one experiment (classical RA) in Group B Table 2 the mononuclear cells were combined with the patient's serum, inactivated at 56° C for 30 minutes. Also here cytotoxicity was provoked.

In some of the cases shown in Table 1 the synovial C activity was also determined. The results are shown in Table 3. In the non rheumatoid controls Group B from Table 1 all three types of tests were negative while in the other groups there is no evident agreement between the three tests.

TABLE 3

*The Cytotoxic Effect of Mononuclear Cells (without Addition of Serum), the Synovial Complement (C) Activity and the Sensitized Sheep Cell (SSC) Test in Different Types of Arthritis*

| Diagnosis                | No. of patients | Post hoc SSC test | Synovial C activity normal or suppressed | Cytotoxic effect |   |        |
|--------------------------|-----------------|-------------------|--|------------------|---|--------|
|                          |                 |                   |  | —                | + | ++     |
| SII                      | 2<br>1          | 2<br>0            | 2<br>1                                   |                  |   | 2<br>1 |
| Felty's syndrome         | 1               | 1                 | 1  | 1                |   |        |
| Classical RA             | 5<br>3          | 5<br>3            | 1<br>3                                   | 4<br>3           | 5 | 3      |
| Definite RA              | 2               | 0                 | 2  | 2                |   |        |
| Non rheumatoid arthritis |                 |                   |  |                  |   |        |
| A Psoriatic arthropathy  | 2               | 0                 | 1  | 1                |   | 2      |
| B Other                  | 10              | 0                 | 10                                       | 10               |   |        |

See Table 1

This limited series does not allow for any detailed clinical considerations. Yet it may be mentioned that two patients with RA with a relatively fresh effusion (for the past two months) showed a negative cytotoxic test. In one of these in whom the disease had lasted for about six months this test was also performed with the addition of (fresh) serum. In the presence of patient serum but not of blood donor serum the test for cytotoxicity became positive. These two were the only ones in whom the disease had lasted for less than one year. In a third case—of suspected SLL—with an effusion existing for the past half year the test for cytotoxicity (performed only with mononuclears without addition of serum) was positive—There was no connection between on the one hand the result of the cytotoxic test and on the other cell

counts and percentage of granulocytes, the amount of synovial fluid aspirated, or the type of treatment

## DISCUSSION

An *in vitro* test has been used for the study of the cytotoxic effect of mononuclear cells obtained from the synovial fluid. Although the mechanism underlying the cytotoxic effect is still unknown, parallels drawn with similar phenomena in experimental autoimmune diseases and some clinical entities suggest that the present test system may be of interest. As reviewed in the introduction, cytotoxic *in vitro* effects have been described in, for instance, homograft rejection, experimental allergic encephalomyelitis, experimental allergic thyroiditis, multiple sclerosis, and ulcerative colitis.

In the present paper, a positive cytotoxic effect has been obtained in tests performed with mononuclear cells from patients with SLE, psoriatic arthropathy, and some cases of RA, whereas tests were negative in all other patients (cf Table 1).

The presence of various immunological disturbances in RA and SLE makes it reasonable to interpret a positive cytotoxic effect as an expression of autoimmunity in these diseases. The positive cytotoxic test in the two patients with psoriatic arthropathy is interesting but will not be further discussed.

In experimental autoimmune diseases, the pathogenetic importance of sensitized lymphocytes has been demonstrated by transfer experiments (cf introduction) and, the appearance of cytotoxic lymphoid cells in the peripheral circulation before the development of the disease (Berg & Hallen 1963) indicates that this phenomenon is due to the immunization procedure. By studies of clinical entities, it is impossible at present to state whether the cytotoxicity is primarily involved or it is secondary to tissue damages.

In experiments with combinations of mononuclear cells and serum in the tests two different types of effect were seen. In five cases, the cytotoxicity of the mononuclears was abolished by addition of serum.

The patient's own serum or blood donor serum. A similar protective effect was seen by Rosenau & Woon (1961) when guinea pig serum was added to cytotoxic lymphocytes in tissue culture. In another three cases, however, the opposite effect was seen. Here the mononuclears without serum proved to be non toxic. After addition of the patient's own serum strong cytotoxicity developed—blood donor serum had no effect in two of these cases. Govaerts (1960) obtained an acceleration of a cytotoxic effect by addition of complement, and Perlmann & Broberger (1963) needed complement to demonstrate a cytotoxic effect of lymphoid cells.

Pulvertaft *et al* (1959) demonstrated a cytotoxic serum factor in Hashimoto's disease, Barnstein & Appel (1961) a myelinolytic serum

factor in experimental allergic encephalomyelitis, and Berg & Kallen (1962) a cytotoxic serum factor in the same disease. Using our system we have found no indications of a cytotoxic effect of RA serum on human fibroblasts. Fraser & Call (1961) gave a preliminary communication on cell damaging effects of RA sera on cultured human synovial cells.

Our findings of a protective or potentiating effect of added serum on the cytotoxicity of the mononuclear cells suggest that several serum factors may be of importance in this connection, and that variations in these factors possibly may influence the cytotoxicity of the mononuclear cells. Paterson (1963) has demonstrated the presence of both cytotoxic (probably 7S) and protective (19S) serum antibodies in rats with experimental allergic encephalomyelitis.

More sensitive methods for evaluating cytotoxicity, e.g., determination of isotope release as suggested by Perlmann & Broberger (1963) or studies on growth carried out by Fraser & Call (1961) might perhaps have produced a higher percentage of positive results in the study of mononuclear cells from patients with arthritis.

## SUMMARY

1 The cytotoxic effect of mononuclear cells from the synovial fluid of patients with various forms of arthritis was *in vitro* tested on human embryonic fibroblasts. Cytotoxicity was judged by "contactual agglutination" and fibroblast degeneration. Only very marked effects were judged as positive.

2 Positive cytotoxicity tests were found in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and two with psoriatic arthropathy. Control subjects with non-rheumatoid arthritis of other types were negative.

3 No absolute agreement between the outcome of the cytotoxicity test, the sensitized sheep cell test, and the suppression of synovial C-complement, could be observed.

4 Parallels are drawn with the presence of similar cytotoxic phenomena in experimental autoimmune diseases and some clinical entities in which an autoimmune pathogenesis has been suggested. The possibility that the cytotoxic phenomenon is secondary to tissue damage in the disease, however, is also stressed.

5 Addition of serum to the medium at the lymphoid cell test may have different effects. A positive cytotoxic reaction can be neutralized by the patient's own, fresh serum or blood donor serum, fresh or inactivated at 56° C for 30 mins. In three instances, a negative cytotoxic effect turned positive after addition of the patient's own fresh serum—blood donor serum had no effect in two cases where it was tested. In one of these, inactivated patient serum also provoked cytotoxicity.

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## EXPERIMENTAL PLATELET AND COAGULATION THROMBI

*A Histological Study of Arterial and Venous Thrombi of  
Varying Age in Untreated and Heparinized Rabbits*

By

LEIF JØRGENSEN

Received 16 v III

The concept of thrombosis as an intravital and intravascular formation of a body derived from blood constituents was introduced by Virchow (1856). Later Zahn (1891), Aschoff (1892), and Welch (1910) gave comprehensive descriptions of the morphology of the thrombus. Until the advent of electron microscopy little has been added to this subject by later authors.

Through the works of Bizzozero (1882) and Eberth & Schimmelbusch (1886 a, b, and c) it was made clear that thrombosis starts with adhesion of platelets to the vascular wall followed by aggregation of additional platelets. The same sequence of events was observed during the haemostatic process (Hayem 1882, Lubnitzky 1885, Eberth & Schimmelbusch 1886 b), with the difference that here the platelets formed a largely extravascular body, the platelet plug. In previous histological studies of haemostasis (Jørgensen & Borchgrevink 1963 a and b 1964) the following stages in the morphological development of the platelet plugs were recognized:

- (1) Loosely aggregated discrete platelets with granular cytoplasm.
- (2) Densely packed granular platelets which often seemed to have fused although the further development indicated that this was not the case.
- (3) Densely packed swollen platelets with an empty or poorly stained interior. The individual platelets could clearly be made out and the aggregates were surrounded by a thin fibrin membrane.
- (4) Apparently more loosely packed somewhat shrunken, discrete platelets surrounded by a thick fibrin membrane.

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tion This was confirmed by the observation that in clotting defects, including heparin treatment the fibrin net after 24 hours was thinner and the meshes wider than normal (*Jorgensen & Borchgrevink 1964*)

In this study the morphological development of platelet aggregates and fibrin net will be reconsidered based on histological examination of experimental thrombi produced in rabbits by two different methods The intention is to elucidate certain pathogenetic mechanisms in the formation and further development of thrombi until they become organized It is felt appropriate to compare the observations in the thrombi with those in the platelet plugs and in platelet aggregates formed *in vitro* Therefore the study will include short reports on the appearance of platelet plugs in rabbits and of aggregates of human platelets formed *in vitro* through the addition to platelet rich plasma of ADP connective tissue tissue thromboplastin calcium and thrombin

An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (*Jorgensen 1964 a*)

## MATERIALS

Animals used were 10 or 12 months old with 0.9 per cent saline 10 or 12% in a few experiments 1:50 or 1:100 A fresh dilution was prepared for each experiment  
 Morris Plains N.J. U.S.A.)  
 10 units per ml (Lipo Heparin)  
 St Louis Mo. U.S.A.)  
 micrograms per ml  
 Hovig (1962) Dr Hovig  
 Human tissue thromboplastin prepared from brains as described by Owren (1949)  
 Thrombin (Topostasin "Roche" Basel Switzerland) dissolved in 0.9 per cent saline to a concentration of 10 NIH units per ml

## METHODS

Experiment 1  
 were used  
 the rabbit  
 during

Anaesthesia was carried out by intravenous amytal sodium 0.04 g per kg body weight supplemented by a mixture of two thirds of ether and one third of 96 per cent ethanol on open mask

Thrombosis was precipitated in the femoral artery or vein by one of the following two methods

- 1 The vessel was carefully dissected on was distal end of the vascular segment

- (5) Diffusely granular masses which take the fibrin stain throughout. The outlines of the shrunken platelets were discerned with difficulty or not at all.

In electron microscope the ballooning of the platelets corresponds to a reduced density of the hyaloplasm and disappearance of the organelles (Kjerheim & Hovig 1962), a process termed degranulation by French & Poole (1963).

From the changes in the haemostatic mechanism in von Willebrand's disease, Jorgensen & Borchgrevink (1964) found it likely that the initial adhesion to the endothelial lips is triggered by adenosine diphosphate (ADP). The subsequent platelet aggregation is precipitated by the same substance (Gaarder, Jonsen, Laland, Hellem & Owren 1961) and connective tissue fibres (Zucker & Borrelli 1962). According to Hovig (1962) the aggregates induced by ADP are looser than those formed by connective tissue "extract". Thus, the loose aggregation in the first stage may be an effect only of ADP. The following stages are probably the result of a continuous formation of thrombin (Jorgensen & Borchgrevink 1963 a and b, 1964). However, degranulation of platelets may also be precipitated by connective tissue (Hovig 1962).

In larger thrombi platelet aggregates form columns or a coralline framework which extends into the vascular lumen (Zahn 1891, Aschoff 1892, Welch 1910). A fibrin net occupies the spaces between the aggregates, and often it forms the major part of the thrombus downstream or upstream to the platelet part. Even in the formation of this net the platelets play an important rôle (Ranvier 1873) Iseri & Benditt (1961), Parmeggiani (1961), and Jorgensen & Borchgrevink (1963 a) stressed the difference in the morphological development of the platelets in larger aggregates and in the small platelet clumps entering into the formation of a fibrin net. In the former case the platelets remain discrete for a long time, even after being engulfed by monocytes (Hand & Chandler 1962). In the latter case the platelets disintegrate and even fuse, also when viewed in electron microscope, forming granular bodies from which the fibrin strands radiate (Rodman, Painter & McDavitt 1963). Jorgensen & Borchgrevink (1964) suggested that the mentioned difference depends on the local concentration of thrombin. In clotting blood this is higher than on the platelet surface within a large platelet aggregate. Ample amounts of thrombin tend to rupture the platelet membranes (Hovig 1962).

In thrombi there is usually a considerable increase in the amount of fibrin with time, not only within the larger platelet aggregates but also in the fibrin net (Aschoff 1892). Jorgensen & Borchgrevink (1963 a) observed the same phenomenon in the hematoma of the wounds made for the primary bleeding time test 24 hours after the bleeding. We considered that this was caused by a prolonged or continuous coagulation of plasma leaking out into the wound, and not only due to clot retrac-

tion This was confirmed by the observation that in clotting defects, including heparin treatment, the fibrin net after 24 hours was thinner and the meshes wider than normal (Jorgensen & Borchgrevink 1964).

In this study the morphological development of platelet aggregates and fibrin net will be reconsidered, based on histological examination of experimental thrombi produced in rabbits by two different methods. The intention is to elucidate certain pathogenetic mechanisms in the formation and further development of thrombi until they become organized. It is felt appropriate to compare the observations in the thrombi with those in the platelet plugs and in platelet aggregates formed *in vitro*. Therefore, the study will include short reports on the appearance of platelet plugs in rabbits, and of aggregates of human platelets formed *in vitro* through the addition to platelet-rich plasma of ADP, connective tissue, tissue thromboplastin, calcium, and thrombin.

An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (Jorgensen 1964 a).

## MATERIALS

Rabbit tissue thromboplastin (Samplastin Warner Morris Plains NJ, U S A )  
 Heparin sodium in aqueous solution 20 000 USP units per ml (Lipo-heparin  
 "Richel", Northridge Calif, U S A )  
 Adenosine diphosphate dihydrate (Sigma Chemical Co, St Louis Mo, U S A )  
 dissolved in 0.9 per cent saline to a concentration of 10 micrograms per ml  
 Saline extract of tendons was prepared as described by Hoots (1982).

## METHODS

Experiment 1. . . . .  
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The blue rumen to about  $\frac{1}{2}$  to  $\frac{3}{4}$  of the original size. Histologically, it was later checked in most cases that the vessel had not been completely occluded by this procedure (Fig 11). About 0.1-0.2 ml of diluted staphylococ-

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In thrombi there is usually a considerable increase in the amount of fibrin with time, not only within the larger platelet aggregates but also in the fibrin net (*Aschoff 1892*) *Jorgensen & Borchgrevink (1963 a)* observed the same phenomenon in the haematoma of the wounds made for the primary bleeding time test 24 hours after the bleeding We considered that this was caused by a prolonged or continuous coagulation of plasma leaking out into the wound, and not only due to clot retraction

tion This was confirmed by the observation that in clotting defects, including heparin treatment, the fibrin net after 24 hours was thinner and the meshes wider than normal (*Jorgensen & Borchgrevink 1964*)

In this study the morphological development of platelet aggregates and fibrin net will be reconsidered based on histological examination of experimental thrombi produced in rabbits by two different methods. The intention is to elucidate certain pathogenetic mechanisms in the formation and further development of thrombi until they become organized. It is felt appropriate to compare the observations in the thrombi with those in the platelet plugs and in platelet aggregates formed *in vitro*. Therefore, the study will include short reports on the appearance of platelet plugs in rabbits and of aggregates of human platelets formed *in vitro* through the addition to platelet rich plasma of ADP, connective tissue, tissue thromboplastin, calcium and thrombin.

An account of the reactions in the vascular wall and of the organization of thrombi will follow in a subsequent paper (*Jorgensen 1964 a*)

## MATERIALS

1. 2.

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Human tissue thromboplastin

1.

1.

1.

Human tissue thromboplastin prepared from brains as described by Owren (1949)

Thrombin (Topostasin, Roche, Basel, Switzerland) dissolved in 0.9 per cent saline to a concentration of 10 NIH units per ml

## METHODS

**Experimental thrombosis in rabbits** Eighty-eight healthy rabbits of albino breed were used. Forty-six were males, 42 females. Their median age was 21 months and their mean weight 3.173 kg. They were kept on a low fat standard diet prior to and during the experiment.

Anaesthesia was carried out by intravenous amytal sodium 0.04 g per kg body weight supplemented by a mixture of two thirds of ether and one third of 96 per cent ethanol on open mask.

Thrombosis was precipitated in the femoral artery or vein by one of the following two methods:

1. The vessel was carefully exposed in about 3 cm of length. No attempt at finer dissection was made and branches or tributaries were not ligated. At the distal end of the exposed arterial segment or at the proximal end of the venous segment a permanent loosely applied ligature of no. 1 silk was placed, constricting the lumen. It was later occluded by



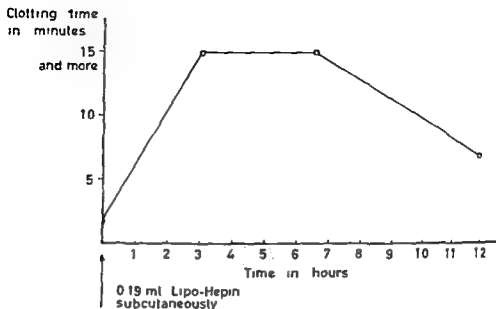


Fig 1

The clotting time in a rabbit after a single subcutaneous injection of heparin when the dose was considered satisfactory

cal  $\alpha$  haemolysin was injected into the ventral side of the vascular wall through several punctures with a no 20 or 22 hypodermic needle. Leakage of toxin into the gap of the wound was inevitable, but direct intravascular injection was avoided.

- (2) The vessel was exposed and a ligature applied as before, but now with complete occlusion of the vessel. About 0.1-0.2 ml of rabbit tissue thromboplastin was injected into the vessel through a no 20 or 22 hypodermic needle. Immediately afterwards the open end of the exposed segment was occluded by a bulldog clamp. After 5 minutes it was removed, the vessel now being filled by a coagulation thrombus which could usually be discerned through the vascular wall.

When heparin treatment was given it was instituted 24 hours after the operation and continued until the end of the experiment. In 2 rabbits the treatment was started half an hour after the operation. The heparin was given subcutaneously every 12 hours in doses which varied from 500 to 1150 LSP units per kg body weight (5.115 mg per kg body weight). The mean dose was 816 LSP units (8.16 mg). The dose was decided individually before the experiment by measuring the whole blood clotting time several times after test doses of heparin. The clotting time was determined by filling a capillary pipette with freely flowing blood from a cut in the ear and breaking off pieces of the pipette at intervals. This method was chosen because it made possible frequent determinations without destroying the ear vessels needed for the anaesthesia. The clotting time was recorded as the time from the filling of the pipette until the first appearance of a fibrin thread. In untreated animals this was usually  $1\frac{1}{2}$  to 2 minutes. The heparin dose was considered satisfactory when the clotting time exceeded twice the normal value. 12 hours after the injection (Fig 1) the preparatory heparin treatment was discontinued for at least 48 hours prior to the precipitation of the thrombus. The effectiveness of the treatment after the operations was checked by random tests; adjustments of the dose were rarely necessary.

In 11 animals one of the methods mentioned was used only on one side (Table 1). In 77 animals staphylococcal  $\alpha$  haemolysin was injected on the left side, tissue thromboplastin on the right. In 22 of these heparin was given; the remaining 66 animals got no further treatment. The rabbits were sacrificed by decapitation from  $\frac{1}{2}$  hour to 50 days after the operation (Table 1). 5 rabbits died in a shocklike state

TABLE 1  
Number of Rabbits Sacrificed at Various Intervals after the Operation

| Number of Rabbits Sacrificed at Various Intervals after the Operation |        |   |      |   |   |   |   |    |    |    |    |    |    |    |       |
|---|--------|---|------|---|---|---|---|----|----|----|----|----|----|----|-------|
| Treatment given   | 1 hour |   | Days |   |   |   |   |    |    |    |    |    |    |    | Total |
|   | 1      | 2 | 3    | 5 | 6 | 7 | 8 | 10 | 12 | 17 | 24 | 70 |    |    |       |
| Non heparinized   |        |   |      |   |   |   |   |    |    |    |    |    |    |    |       |
| Staphylococcal toxin left side - tissue thromboplastin right side     | 4      | 7 | 5    | 2 | 4 | 1 | 4 | 1  | 4  | 7  | 7  | 4  | 5  |    |       |
| Staphylococcal toxin left side  |        | 1 | 1    | - | - | 1 | - | -  | -  | -  | -  | -  | 3  |    |       |
| Tissue thromboplastin right side                                      |        | - | -    | 2 | 1 | 1 | 1 | -  | 1  | 1  | -  | 2  | 8  |    |       |
| Heparinized   |        |   |      |   |   |   |   |    |    |    |    |    |    |    |       |
| Staphylococcal toxin left side - tissue thromboplastin right side     | -      | - | 5    | 4 | 4 | - | 3 | -  | 2  | 4  | -  | -  | 22 |    |       |
| Total   | 4      | 8 | 11   | 8 | 9 | 1 | 9 | 1  | 8  | 8  | 8  | 7  | 6  | 88 |       |

on the second or third day. The femoral vessels with the surrounding tissue were taken out and immediately transferred to Helly's solution or formalin mercuric chloride solution for fixation.

*Experimental haemostasis in rabbits* In 3 rabbits a cut, 1 cm long, was made at the tip of the ear with a razor blade under general anaesthesia. The wounds bled freely, the bleeding times were from 6 to 10 minutes. About 15 minutes after the cessation of the bleeding the cuts with the surrounding tissue were excised and transferred to Helly's solution.

*Human platelet aggregates formed in vitro* Fifty millilitres of blood were mixed with 5 ml trisodium citrate dihydrate 3.1 per cent solution and centrifuged at 275 G for 10 minutes. The platelet-rich plasma was decanted and divided into 5 portions of 2.5 or 4 ml. The following additions were made:

- (1) ADP, 0.3 ml (3 micrograms) to 2.5 ml plasma
- (2) Saline "extract" of tendons, 0.6 ml to 2.5 ml plasma
- (3) Human tissue thromboplastin, 0.4 ml of a dilution 1:100, immediately followed by  $\text{CaCl}_2$  0.4 ml 70 mM to 4 ml plasma. The diluted thromboplastin time was 59 seconds.
- (4)  $\text{CaCl}_2$  0.4 ml 70 mM, immediately followed by ADP, 0.2 ml (2 micrograms) to 4 ml plasma.
- (5) Thrombin, 0.3 ml (3 NIH units) to 2.5 ml plasma.

Immediately after the additions the test tubes were rotated between the palms. After one minute platelet aggregation was obvious to the naked eye. The aggregates were centrifuged at 125 G for 10 minutes, the supernatant plasma or serum decanted and Helly's solution added.

*Histological technique* Among several fixation fluids tried, Helly's solution gave the best results, particularly in fresh thrombi. After 24 hours of fixation the specimens were rinsed in flowing tap water for another 24 hours. With older thrombi nearly equal results were obtained by the formalin mercuric chloride method of Lendrum, Fraser, Slidders & Henderson (1962), combined with fixation for 7 days in 5 per cent aqueous mercuric chloride.

After fixation the femoral vessels were cut transversely in blocks 0.3-0.4 cm long, embedded in paraffin and cut in transverse or longitudinal sections about 5 microns thick at several depths. The following stains were used:

- (1) Masson's haematoxylin erythrosin saffron
- (2) The acid picro Mallory method for staining fibrin (Lendrum 1949)
- (3) The martius scarlet blue (MSB) method of Lendrum, Fraser, Slidders & Henderson (1962)
- (4) Mallory's phosphotungstic acid haematoxylin (PTAH) method

## RESULTS

In this paper the word thrombus comprises every intravascular body derived from blood constituents, including the smallest platelet aggregate provided it was connected with the vascular wall. The staphylococcal  $\alpha$ -haemolysin gave platelet thrombi or mixed platelet and coagulation thrombi; the tissue thromboplastin gave coagulation thrombi. The number of thrombi obtained at different periods of time after the operation is shown in Tables 2 and 7. With staphylococcal toxin non-intentional thrombi often arose in the vessel adjacent to the treated one. The number of these is indicated in brackets, and they are included in the material. Unless otherwise stated, there was no difference between arterial and venous thrombi produced by the same method.

TABLE 2  
*Number of Thrombi Observed at Various Intervals after the Operation in 88 Non Heparinized Hurlers*

| Number of Thrombi Occurring at Various Intervals           |          |      |       |      |          |     |          |      |          |      |            |     |         |     |         |      |         |     |        |
|--|----------|------|-------|------|----------|-----|----------|------|----------|------|------------|-----|---------|-----|---------|------|---------|-----|--------|
| Type of thrombi  | 1/2 hour |      | 1 day |      | 2-3 days |     | 5-6 days |      | 7-8 days |      | 10-12 days |     | 17 days |     | 28 days |      | 50 days |     | Total  |
|  | Art      | Ven  | Art   | Ven  | Art      | Ven | Art      | Ven  | Art      | Ven  | Art        | Ven | Art     | Ven | Art     | Ven  | Art     | Ven |        |
| Staphylococcal toxin                                       |          |      |       |      |          |     |          |      |          |      |            |     |         |     |         |      |         |     |        |
| Thrombi with platelets fibrin completely organized thrombi | 5(3)     | 7(4) | 2(4)  | 6(2) | 1(2)     | 4   | 2(2)     | 4(1) | 2(3)     | 4    | (2)        | 3   | 1(4)    | 1   | (1)     | 2    |         |     | 47(25) |
| No thrombi   | -        | -    | -     | -    | -        | -   | -        | -    | 2        | 1(2) | 3          | 1   | 1(2)    | 2   | 2(1)    | (1)  |         |     | 14(6)  |
| Total  | 5(3)     | 7(4) | 2(4)  | 6(2) | 1(2)     | 4   | 2(2)     | 4(1) | 4(3)     | 5(2) | 3(2)       | 4   | 4(3)    | 3   | 2(2)    | 2(1) |         |     | 58(31) |
| Tissue thromboplastin                                      |          |      |       |      |          |     |          |      |          |      |            |     |         |     |         |      |         |     |        |
| Thrombi with fibrin completely organized thrombi           | 4        | 7    | 4     | 5    | 1        | 4   | 3        | 3    | 5        | 1    | 1          | -   | -       | -   | -       | -    | -       | -   | 28     |
| No thrombi   | -        | -    | -     | -    | -        | -   | -        | -    | -        | 3    | 3          | 4   | 4       | 3   | 3       | 2    |         |     | 22     |
| Total  | 4        | 7    | 4     | 5    | 1        | 4   | 3        | 3    | 5        | 5    | 4          | 4   | 4       | 3   | 3       | 2    |         |     | 50     |

Figures in brackets indicate non intentional thrombi in the vessel adjacent to the treated one

on the second or third day. The femoral vessels with the surrounding tissue were taken out and immediately transferred to Helly's solution or formalin mercuric chloride solution for fixation.

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- (1) ADP 0.3 ml (3 micrograms) to 2.5 ml plasma
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diameter up to 2-3 microns (Fig. 4). Usually the last mentioned aggregates were equipped with a more or less distinct fibrin membrane at the surface or near and parallel to it. In aggregates with the membrane buried at a certain depth the platelets lying outside tended to be more swollen than those inside (Fig. 4). Beginning at 24 hours a fourth stage in the development of the aggregates was observed: more or less compact masses of dense small platelets with a diameter of less than 1-2

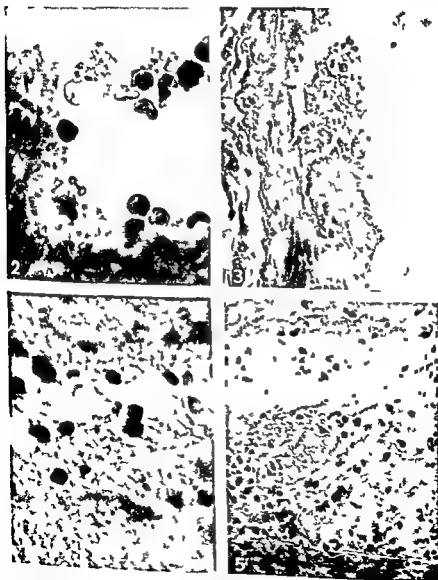


Fig. 4, 5

TABLE 3

*Types of Thrombi Produced by Staphylococcal  $\alpha$  Haemolysin in Von Heparinized Rabbits*

| Type of thrombi  | 1 hour |     | 13 days |     | 54 days |     | 8 days |     |
|--|--------|-----|---------|-----|---------|-----|--------|-----|
|  | Art    | Ven | Art     | Ven | Art     | Ven | Art    | Ven |
| Loose aggregates connected with the wall                       | 2      | 2   | —       | 1   |         | 1   |        | 1   |
| Non occluding mainly platelet thrombi in a more advanced stage | —      | —   | 5       | 2   | 1       | 2   | —      | —   |
| Occluding mixed thrombi  |        | 1   | 7       | 13  | 2       | 1   | 4      | 4   |
| Total  | 2      | 3   | 12      | 16  | 3       | 4   | 4      | 5   |

Intentional and non intentional thrombi are pooled

*Platelet Thrombi and Platelet Part of Mixed Thrombi Made by Staphylococcal  $\alpha$ -Haemolysin in Non-Heparinized Rabbits*

Invariably, injection of staphylococcal toxin resulted in coagulation necrosis of the vascular wall and the surroundings, often including the neighbouring vessel. After the first days a zone of leucocytes formed a characteristic band around the necrotic area. The thrombi varied considerably and were observed in all stages of their development. A common feature of unorganized thrombi was the presence of platelet aggregates. In 7 instances only small, loose clumps of granular platelets occurred, loosely connected with the vascular wall (Fig 2) (Table 3). In several other vessels larger aggregates were well fixed to the wall. They were either composed of densely packed platelets, often apparently fused (Fig 3), or of slightly swollen, degranulated platelets with a

Figs 2-5

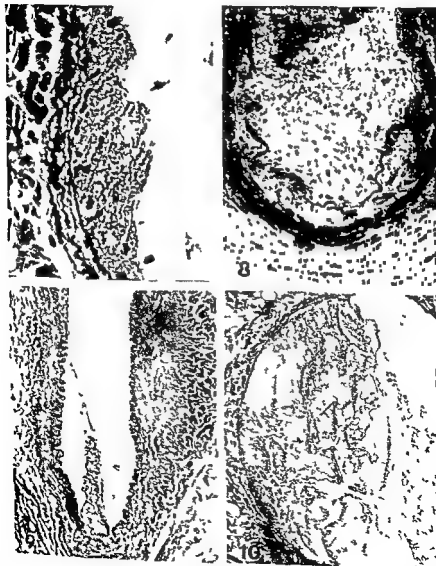
Fig 2 A few loosely aggregated granular platelets adhere to the vascular lining. At the top a small detached platelet clump. Femoral vein beside artery treated with staphylococcal toxin  $\frac{1}{2}$  hour after the operation. Haematoxylin erythrosin saffron  $\times 900$ .

Fig 3 Platelet thrombus with densely packed granular platelets. At upper right a small detached platelet clump. Femoral vein treated with staphylococcal toxin 24 hours after the operation. Lendrum's stain  $\times 260$ .

Fig 4 Part of a coralline platelet thrombus with slightly swollen and degranulated platelets and peripheral fibrin membrane. The platelets are more swollen outside the fibrin membranes (in the middle) than inside (at the top and bottom). Femoral vein treated with staphylococcal toxin 24 hours after the operation. Lendrum's stain  $\times 800$ .

Fig 5 Part of a coralline thrombus with compact masses of small granular platelets interspersed with fibrin net. Femoral artery beside vein treated with staphylococcal toxin 2 days after the operation. Haematoxylin erythrosin saffron  $\times 260$ .

downstream to the stenosing ligature (Fig 11) The large aggregates had at times the shape of perpendicular platelet columns spaced out at intervals (Fig 12) These columns were twisted, bended, and branched, occasionally thrown into an irregular pile The classic coralline structure appeared when the columns were connected with bars of platelet masses parallel to the wall The perpendicular columns tended to widen towards the open lumen, and at the luminal surface the platelet throm



*Figs 7 10*



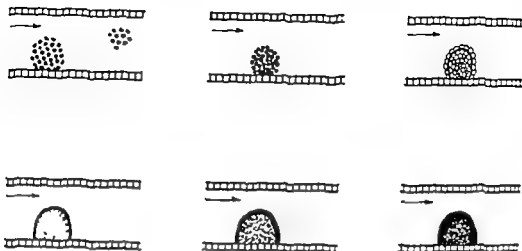


Fig 6

Diagram summarizing the morphological changes of platelet thrombi

microns (Fig 5) These aggregates differed from the early ones by their larger size and their well-defined perimetric fibrin membrane. From the same time several of the aggregates were in various stages of fibrinous transformation: the fibrin membrane broadened, and fibrin strands precipitated between the platelets (Fig 7). As the amount of fibrin increased, the platelets were concealed, resulting in a fibrin mass with a granular appearance. However, aggregates in the middle of mixed thrombi often failed to go through the fibrinous transformation, even after 50 days (Fig 8). The sequence of changes of the aggregates is summarized in Fig 6. A variable amount of white and red blood cells, particularly granulocytes, was seen in the aggregates at all stages.

The small and medium-sized aggregates were often flat, clinging to the vascular wall (Figs 3 and 7). Occasionally, they were erected perpendicular to it (Fig 9), or formed whorl-like structures, particularly

#### Figs 7-10

- Fig 7 Platelet thrombus in which fibrin has precipitated nearly concealing all the platelets. Femoral vein treated with staphylococcal toxin 2 days after the operation. MSB  $\times 300$ .
- Fig 8 Mixed thrombus with a peripheral zone of compact aggregates of small platelets outlined by a distinct wavy fibrin membrane. The delicate net partly lysed. A few shadows of erythrocytes remain. Femoral artery beside vein treated with staphylococcal toxin 50 days after the operation. PTAH  $\times 120$ .
- Fig 9 Platelet column perpendicular to the wall. A fibrin membrane from the tip of the column to the wall probably heralds the precipitation of a coagulation part. Femoral artery beside vein treated with staphylococcal toxin 24 hours after the operation. Landrum's stain  $\times 25$ .
- Fig 10 Coralline platelet thrombus with widening columns and bars towards the open lumen. At the bottom a fragment is on the verge of breaking off. Femoral vein treated with staphylococcal toxin 24 hours after the operation. MSB  $\times 25$ .

bus often terminated in a particularly thick bar (Fig 10) Usually two or more of the mentioned developmental stages were represented in the same aggregate and the youngest parts were always located nearest the lumen Here fragments of the aggregates were commonly on the verge of breaking off from non occluding thrombi (Fig 10) and detached masses could be seen in open lumen (Figs 2 and 3) This which is indicative of an active thrombotic process was not only observed in the first days but even in 1 arterial thrombus after 28 days and in 7 venous thrombi after 5 to 50 days (Fig 13) One artery observed after 17 days deserves special mention Here a defect in the media had prompted the precipitation of dense granular crumbling aggregates without fibrin (Fig 14)

The platelet thrombi were located on both sides of the stenosing ligature but the favourite sites were downstream to it and just at the stenosis (Fig 11) Platelet massing was likewise apt to occur behind venous leaflets

As to the size of the aggregates Table 4 indicates a difference between arterial and venous thrombi It seems to be a tendency for platelet aggregates to develop more slowly in arteries than in veins Thus there was no large aggregate in arteries earlier than after 2 days while 5 of 8 aggregates in veins after 24 hours were of this type All 3 large arterial aggregates after 2 and 3 days were non intentional whereas there was no trend in the distribution of venous aggregates according to size when the figures are split into intentional and non intentional thrombi The largest aggregates were found in non intentional arterial thrombi after the 2nd day

Four vessels treated with staphylococcal toxin and 17 vessels treated with tissue thromboplastin all with restored circulation via collaterals or past old endothelialized lesions contained freely floating platelet aggregates the forming site of which could not be determined (Figs 15 and 16) Usually they were small and loose occasionally larger dense and granular without fibrin In a few instances they had coalesced into conglomerates of discrete or connected granular aggregates with a vague fibrin membrane and a fringe of ballooned platelets (Fig 17)

#### *Coagulation Part of Mixed Thrombi Made by Staphylococcal a Haemolysin and Coagulation Thrombi Made by Tissue Thromboplastin in Non Heparinized Rabbits*

The first sign of clotting was the precipitation of fibrin membranes fixed to platelet thrombi or the vascular wall (Fig 9) In the completed coagulation thrombus the membranes formed a coarse framework (Figs 11 and 18) This included 1 peripheral membrane and in the mixed thrombi one or more parallel membranes within the thrombus running in a streamline pattern In both types of thrombi arched and wavy membranes branched off from the peripheral one or from plate



Figs 11-19

**Fig 11** Mixed thrombus on either side of the stenosing ligature. Flows from left to right. Darker grey areas at the stenosis and downstream to it are platelet parts. Streamline pattern of fibrin membrane in the coagulation part. Femoral vein treated with staploloc at vein 2 days after the operation. Tendril's stain  $\times 25$ .

**Fig 12** Mixed thrombus with parallel lumens spaced out at intervals. Flow from left to right. Femoral vein treated with staploloc at vein 6 days after the operation. Tendril's stain  $\times 25$ .

bus often terminated in a particularly thick bar (Fig 10) Usually, two or more of the mentioned developmental stages were represented in the same aggregate, and the youngest parts were always located nearest the lumen Here fragments of the aggregates were commonly on the verge of breaking off from non occluding thrombi (Fig 10), and detached masses could be seen in open lumen (Figs 2 and 3) This, which is indicative of an active thrombotic process was not only observed in the first days, but even in 1 arterial thrombus after 28 days, and in 7 venous thrombi after 5 to 50 days (Fig 13) One artery observed after 17 days deserves special mention Here a defect in the media had prompted the precipitation of dense, granular, crumbling aggregates without fibrin (Fig 14)

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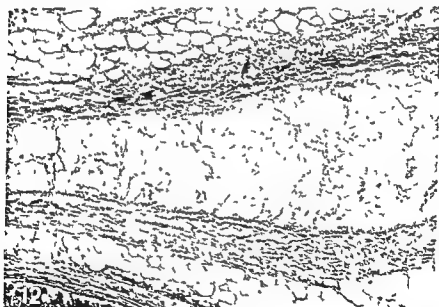
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Figs 11 12

*Fig 11* Mixed thrombus on either side of the stenosing ligature. Flow from left to right. Darker grey areas at the stenosis and downstream to it are platelet parts. Streamline pattern of fibrin membranes in the coagulation part. Femoral vein treated with staphylococcal toxin 2 days after the operation. Iendrum's stain  $\times 25$ .

*Fig 12* Mixed thrombus with parallel columns spaced out at intervals. Flow from left to right. Femoral vein treated with staphylococcal toxin 6 days after the operation. Iendrum's stain  $\times 25$ .

few platelets, and measured 5-15 microns in the freshest thrombi. Larger aggregates with granular or slightly degranulated platelets were intimately connected with the fibrin membranes, not rarely squeezed between two adjacent ones (Fig 19). At the upstream end of thrombi, and at the mouths of venous tributaries, aggregates of the same type as those described in the preceding paragraph were encountered on the surface of the thrombi.

TABLE 4

*Occurrence of Small or Medium Sized vs Large Platelet Thrombi or Platelet Part of Sized Thrombi Produced by Staphylococcal  $\alpha$  Haemolysin*

| Type of platelet aggregates | Arterial thrombi   |       |          | Venous thrombi     |       |          |
|-----------------------------|--------------------|-------|----------|--------------------|-------|----------|
|                             | $\frac{1}{2}$ hour | 1 day | 2-3 days | $\frac{1}{2}$ hour | 1 day | 2-3 days |
| Small and medium sized      | 2                  | 6     | 3        | 3                  | 3     | II       |
| Large                       | 0                  | 0     | 3        | 0                  | 5     | 2        |
| Total                       | 2                  | 6     | 6        | 3                  | 8     | 8        |

Intentional and non intentional thrombi are pooled

Fisher exact probability test ( $\frac{1}{2}$  hour and 1 day grouped together)

Arterial thrombi  $P=0.11$  (two tailed)

Venous thrombi  $II=0.67$  (two tailed)

As the platelet thrombus, the coagulation variety undergoes profound changes with time. In the toxin produced thrombi the erythrocytes were mostly haemolyzed already after 24 hours, while in the thromboplastin made thrombi this was the case only to a slight extent even in the oldest unorganized remnants. From the 2nd day the leucocytes showed elongation of the nuclei, pyknosis and karyorrhexis. Any open space around the thrombus widened. The amount of fibrin increased steadily until fibrinolysis supervened, particularly corresponding to platelet rich parts of the coagulation thrombus. The platelet aggregates along the membranes underwent fibrinous transformation.

Figs 13-16

Fig 13 Active platelet thrombus on a thickened vascular wall. Femoral vein treated with staphylococcal toxin. II days after the operation. Lendrum's stain  $\times 105$ .

Fig 14 Crumbling very dense granular platelet thrombus in a defect of the media accidentally caused during the operation. Femoral artery beside vein treated with staphylococcal toxin. 17 days after the operation. Lendrum's stain  $\times 105$ .

Fig 15

$\alpha$  Staphylococcal

Fig 16 4-5 days after the operation. Lendrum's stain  $\times 105$ . Freely floating conglomerate of granular aggregates with a fringe of ballooned platelets. Femoral artery treated with staphylococcal toxin. 12 days after the operation. Heparinized animal MSR  $\times 22$ .

let aggregates in the mixed type. Frequently, they lined channels within the thrombus, filled by unclotted blood. However, most of the spaces between the membranes were filled by a delicate fibrin net in which red and white blood cells were entrapped, the latter particularly along the membranes, including those of the platelet thrombi. In some of the intersections of the net finely granular bodies appeared as knots and stained as fibrin (Fig 19). They were probably derived from one or a



Figs 13 16

few platelets and measured 5-15 microns in the freshest thrombi. Larger aggregates with granular or slightly degranulated platelets were intimately connected with the fibrin membranes, not rarely squeezed between two adjacent ones (Fig 19). At the upstream end of thrombi and at the mouths of venous tributaries, aggregates of the same type as those described in the preceding paragraph were encountered on the surface of the thrombi.

TABLE 4

*Occurrence of Small or Medium Sized vs Large Platelet Thrombi or Platelet Part of Vized Thrombi Produced by Staphylococcal  $\alpha$  Haemolysin*

| Type of platelet aggregates | Arterial thrombi |       |          | Venous thrombi |       |          |
|-----------------------------|------------------|-------|----------|----------------|-------|----------|
|                             | 1/2 hour         | 1 day | 2-3 days | 1/2 hour       | 1 day | 2-3 days |
| Small and medium sized      | 2                | 6     | 3        | 3              | 3     | 8        |
| Large                       | 0                | 0     | 3        | 0              | 5     | 11       |
| Total                       | 2                | 6     | 6        | 3              | 8     | 8        |

Intentional and non intentional thrombi are pooled  
Fisher exact probability test (1/2 hour and 1 day grouped together)

Arterial thrombi  $P=0.11$  (two tailed)

Venous thrombi  $P=0.67$  (two tailed)

As the platelet thrombus, the coagulation variety undergoes profound changes with time. In the toxin produced thrombi the erythrocytes were mostly haemolyzed already after 24 hours, while in the

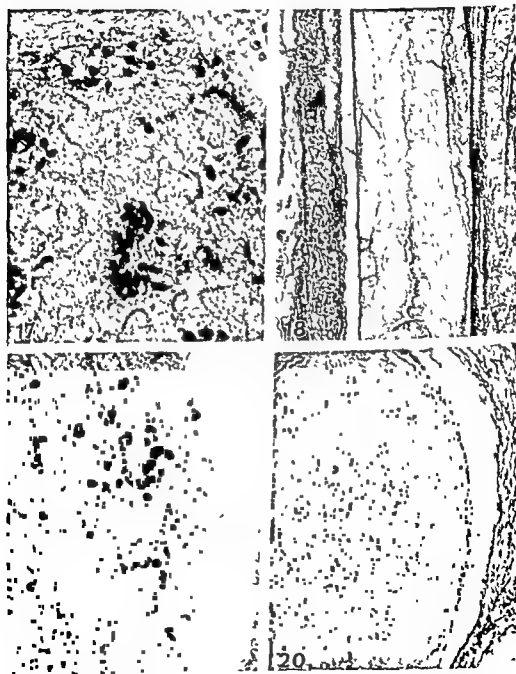
space around the thrombus widened. The amount of fibrin increased steadily until fibrinolysis supervened particularly corresponding to platelet rich parts of the coagulation thrombus. The platelet aggregates along the membranes underwent fibrinous transformation.

Figs 18-16

- Fig 13 Active platelet thrombus on a thickened vascular wall. Femoral vein treated with staphylococcal toxin 17 days after the operation. Lendrum's stain  $\times 105$ .
- Fig 14 Crumbling very dense granular platelet thrombus in a defect of the media accidentally caused during the operation. Femoral artery beside vein treated with staphylococcal toxin 17 days after the operation. Lendrum's stain  $\times 105$ .
- Fig 15 Freely floating aggregate of granular platelets caught in a narrow flow from left femoral artery 7 days after the operation. Heparinized animal. MSB  $\times 22$ .
- Fig 16 Freely floating conglomerate of granular aggregates with a fringe of ballooned platelets. Femoral artery treated with staphylococcal toxin 12 days after the operation. Heparinized animal. MSB  $\times 22$ .



and the knots of platelet origin tended to enlarge and fuse. Especially in the mixed thrombi also the membranes and the delicate net became coarser. The red blood cells, haemolyzed or not, were strangulated and finally concealed by the fibrin net. At this stage the fibrin appeared as broad hyaline bands or masses with small clear spaces in which elongated leucocyte nuclei lingered for a while (Fig 20). At this stage fibrin which had buried the coagulation part of the mixed thrombi could not



Figs 17-20

be distinguished from the fibrin which concealed the platelet part

The increase of fibrin was most marked at the upstream end of the thrombi and in the pure coagulation thrombi, at the injection site. In the middle of the occluding mixed thrombi the increase was only slight, and here the stroma of laked erythrocytes shrank and disappeared, causing collapse of the fibrin net.

In the toxin produced thrombi complete occlusion of the vessel was effected by the coagulation part, and not by the platelet part alone. However, the proportion of the two components varied considerably. Even in a few non-intentional arterial thrombi the platelet aggregation part was rather small. As seen in Table 5 all 11 non-intentional venous thrombi observed after 1-8 days were occluding, while this was the case in only 5 of 11 non-intentional arterial thrombi. This is a reflection of the fact that precipitation of the coagulation part takes place more readily in veins than in arteries. Within either category of vessel the relative frequency of occluding and non-occluding thrombi varied at the different points of time, but it did not show any definite trend after the first day (Table 3). The concentration of the injected toxin did not seem to influence this ratio.

TABLE 5  
*Non Occluding vs. Occluding Non Intentional Arterial  
and Venous Thrombi Produced by Staphylococcal  $\alpha$  Haemolysin  
Observed between 1 and 8 Days after the Operation*

| Type of thrombi | Arterial | Venous |
|-----------------|----------|--------|
| Non Occluding   | 6        | 0      |
| Occluding       | 5        | 6      |
| Total           | 11       | 6      |

Fisher exact probability test  $P=0.075$  (two tailed)

*Figs 17-20*

*Fig 17* Detail of Fig 16 MSB  $\times 260$

*Fig 18* Coagulation part of mixed thrombus with membranous framework including a peripheral membrane. Femoral vein treated with staphylococcal toxin 2 days after the operation. Lendrum's stain  $\times 13$

*Fig 19* Coagulation thrombus with platelet aggregates between ...

*Fig 20* ... with hyaline fibrin masses near the upstream end ... of the clear spaces contain a leucocyte in degeneration. Femoral vein beside artery treated with staphylococcal toxin 7 days after the operation. Lendrum's stain  $\times 105$

TABLE 6  
*Signs of Spontaneous General Fibrinolysis in Mixed Thrombi Made by Staphylococcal or Haemolysin  
 and in Congulation Thrombi Made by Tissue Thromboplastin*

| Type of thrombi               | 1/2 hour |     | 1 day |     | 2-3 days |     | 5-6 days |     | 7-8 days |     | 10-12 days |     |
|-------------------------------|----------|-----|-------|-----|----------|-----|----------|-----|----------|-----|------------|-----|
|                               | Art      | Ven | Art   | Ven | Art      | Ven | Art      | Ven | Art      | Ven | Art        | Ven |
| <i>Staphylococcal toxin</i>   |          |     |       |     |          |     |          |     |          |     |            |     |
| Without signs of fibrinolysis | 3        | 7   | 4     | 7   | 2        | -   | -        | -   | 1        | 2   | 5          | 7   |
| With signs of fibrinolysis    | -        | -   | -     | -   | -        | 1   | -        | -   | 3        | 2   | 2          | -   |
| <i>Tissue thromboplastin</i>  |          |     |       |     |          |     |          |     |          |     |            |     |
| Without signs of fibrinolysis | 4        | 7   | 4     | 4   | -        | -   | -        | -   | 2        | 1   | 1          | 4   |
| With signs of fibrinolysis    | -        | -   | -     | 1   | 1        | 4   | -        | -   | 1        | 2   | 4          | -   |
| No thrombi                    | -        | -   | -     | -   | 1        | -   | -        | -   | -        | -   | -          | 1   |

Intentional and non intentional mixed thrombi are pooled

TABLE 7  
Number of Thromboses at Various Intervals after the Operation in 22 Heparinized Rabbits

| Type of thrombosis       | 1-2 days |       | 3-7 days |   | 8-14 days |   | 15-21 days |   | Total  |
|--------------------------|----------|-------|----------|---|-----------|---|------------|---|--------|
|                          | A        | B     | A        | B | A         | B | A          | B |        |
| Arterial thrombosis      | 3 (2)    | 4 (1) | 1        | 1 | 1 (1)     | 2 | 1 (1)      | 1 | 15 (5) |
| Arteriovenous thrombosis | 1        | 1     | 1        | 1 | 1         | 1 | 1          | 1 | 8      |
| Total                    | 4 (2)    | 5 (1) | 2        | 2 | 1 (1)     | 3 | 3 (1)      | 2 | 23 (5) |
| Arterial thrombosis      | 4        | 5     | 1        | 2 | 1         | 2 | 1          | 1 | 16     |
| Arteriovenous thrombosis | 1        | 1     | 1        | 1 | 1         | 1 | 3          | 1 | 10     |
| Total                    | 5        | 6     | 2        | 3 | 2         | 3 | 4          | 2 | 26     |

Figures in parentheses indicate number of rabbits in the series adjacent to the treated one. In 2 rabbits killed after 2 and 7 days the parin treatment was started 2 days after the operation.

TABLE 6  
*Signs of Spontaneous General Fibrinolysis in Mixed Thrombi Made by Staphylococcal  $\alpha$ -Haemolysin  
 and in Coagulation Thrombi Made by Tissue Thromboplastin*

| Type of thrombi               | 1st hour |     | 2-3 days |     | 5-6 days |     | 7-8 days |     | 10-12 days |     |
|-------------------------------|----------|-----|----------|-----|----------|-----|----------|-----|------------|-----|
|                               | Art      | Ven | Art      | Ven | Art      | Ven | Art      | Ven | Art        | Ven |
| <i>Staphylococcal toxin</i>   |          |     |          |     |          |     |          |     |            |     |
| Without signs of fibrinolysis | 3        | 7   | 4        | 7   | 2        | -   | 1        | 2   | 5          | 7   |
| With signs of fibrinolysis    | -        | -   | -        | -   | -        | 1   | 3        | 2   | 2          | -   |
| <i>Tissue thromboplastin</i>  |          |     |          |     |          |     |          |     |            |     |
| Without signs of fibrinolysis | 4        | 7   | 4        | 4   | -        | -   | 2        | 1   | 1          | 4   |
| With signs of fibrinolysis    | -        | -   | -        | 1   | 1        | 4   | 1        | 2   | 4          | -   |
| No thrombi                    | -        | -   | -        | -   | 1        | -   | -        | -   | -          | 1   |

Intentional and non intentional mixed thrombi are pooled

5th day in the thromboplastin made thrombi. It was more pronounced than usual particularly in venous—and thromboplastin made—thrombi. Among unorganized thrombi only 2 of the mixed type and 1 of the coagulation type lacked any sign of fibrinolysis.

As in non heparinized animals vessels with restored circulation contained young freely floating platelet aggregates or conglomerates of such aggregates (Figs 16 and 17). However, they were more frequent in heparinized animals. 11 vessels treated with staphylococcal toxin and 10 treated with tissue thromboplastin showed these aggregates, i.e., nearly half of the vessels. As stated above, the corresponding figures in non heparinized animals were 4 and 17, i.e., about one sixth of the vessels. But this comparison is not quite fair because the aggregates in the heparinized animals were seen even in the first days, at a time when in non heparinized animals the circulation was often completely blocked by unlysed thrombi. Nevertheless within each vessel of the heparinized animals the aggregates were more numerous and larger than usual.

### *Platelet Plugs in Rabbits*

Platelet plugs were not only observed in the ear wounds of the 3 rabbits but also at the puncture site in several of the vessels treated with tissue thromboplastin. In most respects they were identical to those observed in man (*Jorgensen & Borchgrevink* 1963 a and b). However, their morphological development was slower. Fifteen minutes after the bleeding the plugs had often not reached the stage of ballooning and those who had showed less swelling of the platelets than many human plugs at the same time (Fig 24). The marked ballooning of the platelets was obtained in some of the puncture site plugs at 24 hours (Fig 25). The fibrinous transformation was completed after 2 days, sometimes after 24 hours.

### *Human Platelet Aggregates Formed in vitro*

ADP precipitated small loose collections of granular platelets (Fig 26). Morphologically they were indistinguishable from (1) those observed as the first stage of platelet thrombi, (2) many of the young freely floating aggregates the origin of which could not be traced, and (3) the platelet aggregates covering the mouths of the bleeding vessels in wounds with severe clotting defects (*Jorgensen & Borchgrevink* 1961).

Saline extract of tendons gave at the same point of time larger, irregularly formed aggregates with varying density (Fig 27). Platelets were closely clumped together often obscuring the outlines of the individual platelet. In between platelets were more loosely arranged. Their cytoplasm was granular and dense with the exception of a few scattered degranulated platelets. No fibrin was present. The picture had

stream (Fig 8) In general, the fibrinolysis was more marked in veins than in arteries, and more marked in the thromboplastin-made thrombi than in the toxin-produced A consequence of this is the fact that arterial thrombi, and toxin-produced, healed with more extensive permanent sequelae than their respective counterparts (Jørgensen 1964 a)

### Heparin Treatment

Heparin medication after the initiation of thrombosis by staphylococcal toxin reduced the number of vessels with thrombi from 57 of 58 (98 per cent) to 17 of 22 (77 per cent) (Tables 2 and 7) Further, the number of non-intentional thrombi decreased from 31 in 58 non-injected vessels (53 per cent) to 5 in 22 (23 per cent) These differences are below the conventional level of significance when tested by the  $\chi^2$  method ( $P < 0.01$  and  $0.05 > P > 0.02$  respectively) There was no difference in the occurrence of coagulation thrombi produced by tissue thromboplastin whether heparin was given or not

TABLE 8  
*Signs of General Fibrinolysis under Heparin Treatment  
in Mixed Thrombi Made by Staphylococcal  $\alpha$  Haemolysin and in Coagulation  
Thrombi Made by Tissue Thromboplastin*

| Type of thrombi               | 2-3 days |     | 5 days |     | 7 days |     | 10-12 days |     |
|-------------------------------|----------|-----|--------|-----|--------|-----|------------|-----|
|                               | Art      | Ven | Art    | Ven | Art    | Ven | Art        | Ven |
| <i>Staphylococcal toxin</i>   |          |     |        |     |        |     |            |     |
| Without signs of fibrinolysis |          | -   | 1      |     |        | 1   | 1          | 2   |
| With signs of fibrinolysis    | 4        | 4   | 1      | 1   | 2      |     | 2          | 1   |
| No thrombi                    | 1        | 1   |        | 1   |        | 1   | -          | 1   |
| <i>Tissue thromboplastin</i>  |          |     |        |     |        |     |            |     |
| Without signs of fibrinolysis | -        | 2   | -      | 1   | -      | 1   | 1          | 3   |
| With signs of fibrinolysis    | 4        | 3   | 1      | 1   | 1      | 1   | 2          |     |
| No thrombi                    |          |     | 1      | -   |        |     | -          |     |

Intentional and non intentional mixed thrombi are pooled

Two of the heparinized toxin-produced thrombi after 2 and 11 days consisted only of small loose platelet aggregates connected with the wall The remaining thrombi made by this method were mixed thrombi The fibrin amount of most thrombi was not or only slightly less than that of the non-heparinized Only 2 mixed and 2 coagulation thrombi in 4 different rabbits killed after 5 days or earlier had obviously a more delicate and wide meshed fibrin net than usual Much more conspicuous was the tendency towards general fibrinolysis (Fig 22) (Table 8) It started already after 2 days and, due to progressing organization, subsided from the 10th day in the toxin produced thrombi, from the

5th day in the thromboplastin made thrombi. It was more pronounced than usual particularly in venous—and thromboplastin made—thrombi. Among unorganized thrombi only 2 of the mixed type and 2 of the coagulation type lacked any sign of fibrinolysis.

As in non heparinized animals, vessels with restored circulation contained young freely floating platelet aggregates or conglomerates of such aggregates (Figs 16 and 17). However they were more frequent in heparinized animals. 11 vessels treated with staphylococcal toxin and 10 treated with tissue thromboplastin showed these aggregates, i.e. nearly half of the vessels. As stated above the corresponding figures in non heparinized animals were 4 and 17, i.e. about one sixth of the vessels. But this comparison is not quite fair because the aggregates in the heparinized animals were seen even in the first days at a time when in non heparinized animals the circulation was often completely blocked by unlysed thrombi. Nevertheless within each vessel of the heparinized animals the aggregates were more numerous and larger than usual.

### *Platelet Plugs in Rabbits*

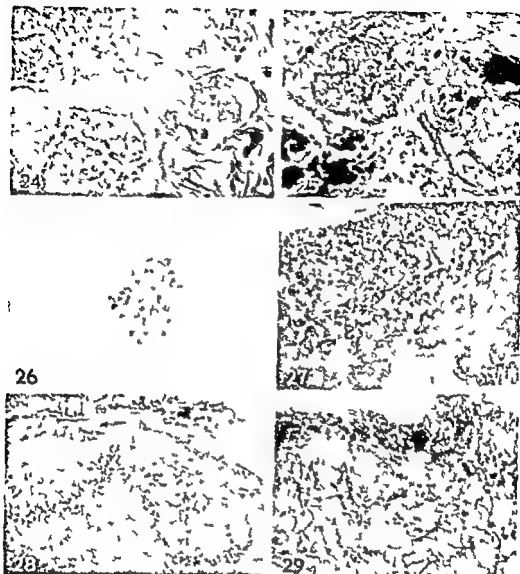
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ADP precipitated small loose collections of granular platelets (Fig 26). Morphologically they were indistinguishable from (1) those observed as the first stage of platelet thrombi, (2) many of the young freely floating aggregates the origin of which could not be traced, and (3) the platelet aggregates covering the mouths of the bleeding vessels in wounds with severe clotting defects (Jørgensen & Borchgrevink 1964).

Saline "extract" of tendons gave at the same point of time larger irregularly formed aggregates with varying density (Fig 27). Platelets were closely clumped together often obscuring the outlines of the individual platelet. In between platelets were more loosely arranged. Their cytoplasm was granular and dense with the exception of a few scattered degranulated platelets. No fibrin was present. The picture had





Figs 24-29

- Fig 24 Illig with slightly ballooned platelets in front of a capillary vein. Farwood in a rabbit 15 minutes after the heparin. MSB  $\times 240$
- Fig 25 Illig with more marked ballooning of the platelets at the periphery of a vein treated with tissue thromboplastin 24 hours after the operation. Lencrum  $\times 240$
- Fig 26 Loosely collected granular platelets from cells after intravenous haemolysis in erythrosin saffron  $\times 800$
- Fig 27 Platelet aggregate of varying density many platelets still clumped together. Produced by saline extract of testis. ITAH  $\times 30$
- Fig 28 Platelet aggregate with a granular centre and a few cells on platelets outside a faintly indicated fibrin membrane from cells in tissue thromboplastin. MSB  $\times 320$
- Fig 29 Densely packed platelet aggregate in a delicate net of fibrin with several black knots of platelet origin. Produced by thrombin. ITAH  $\times 320$

no great likeness to any aggregate observed in experimental thrombi or haemostasis perhaps except the thrombus shown in Fig 14

Tissue thromboplastin and recalcification plus ADP gave dense granular aggregates with a faintly indicated fibrin membrane near and parallel to the surface (Fig 28) Platelets lying outside this membrane were clearly swollen and degranulated The thrombin induced aggregates were similar but they lacked the characteristic fibrin membrane and the ballooned platelets (Fig 29) The former type of aggregates were identical to those of the freely floating conglomerates whereas the latter type resembled the granular aggregates connected with the fibrin membranes of coagulation thrombi By all three methods a young delicate fibrin net had precipitated containing knots of platelet origin smaller than the granular bodies of the coagulation thrombi (Fig 29)

Aggregates with ballooning of platelets throughout as in naturally occurring platelet plugs and thrombi could not be precipitated by any of the methods

### DISCUSSION

In the pathogenesis of thrombosis three sets of predisposing factors have repeatedly been discussed viz. lesions of the vascular wall mechanical disturbances of the circulation and alteration of the blood (Virchow 1856 Welch 1910 Aschoff 1938 Poole & French 1961 Vinograd Murphy Rowse & Downie 1962) In this experimental study these factors have been combined in two different ways By injection of staphylococcal toxin and partial ligation all three factors became involved in so far as vascular necrosis slowing and edding of the blood flow and local hypercoagulability were created By injection of tissue thromboplastin and occluding ligation mainly alterations in the circulation and blood were produced Of course the pathogenesis of the thrombi formed may be different from most naturally occurring thrombi in man and the present observations cannot be generally applied with reservations

The preparation of staphylococcal toxin contained fairly large amounts of  $\alpha$ -haemolysin as opposed to other haemolysins (Onland 1955) Other staphylococcal toxins are assumed to be present but this has not been tested (Onland 1964) The presence of necrotizing dermatin however is evident from the wide spread necrosis identical to that observed in human infections (Larsen & Jorgensen 1960) Production of coagulase is usually associated with that of  $\alpha$ -haemolysin (Eick & Leis 1950) Nevertheless with the severe vascular lesions created here it is likely that a significant amount of thrombin is also produced via usual mechanisms i.e. the extrinsic clotting system triggered by tissue thromboplastin and the intrinsic clotting system where this is started by activation of Hageman factor (Factor XII) tissue thromboplastin (Wanler 1957) or extracellular tissue fluid (Guren 1962)

In spite of this, the initial step in the toxin-produced thrombosis in flowing blood was not fibrin precipitation, but platelet adhesion and aggregation. In accordance with the electronmicroscopical findings of *Poole, French & Cliff* (1963) the earliest platelet thrombi consisted of loose aggregates of granular platelets. They were morphologically identical to the platelet plugs in wounds where the clotting was most impaired (*Jorgensen & Borchgrevink* 1964). The same type of aggregates was produced *in vitro* by the addition of ADP to platelet-rich plasma confirming the electronmicroscopical observations by *Hovig* (1962). Likewise in conformity with *Hovig* (1962), aggregates formed by tendon "extract" were, by the same time, generally more dense. But the density varied, which gave the masses a clumpy appearance, unlike most platelet aggregates in thrombosis or haemostasis. These findings are compatible with the concept that it is ADP, and not connective tissue, which brings about the initial aggregation. If so, it is also likely that the initial adhesion of the platelets to the damaged wall is precipitated by ADP, as previously indicated in the case of human haemostasis (*Jorgensen & Borchgrevink* 1964). *Honour & Mitchell* (1963) demonstrated *in vivo* that application of ADP to the vascular wall prompted platelet thrombosis but, in higher concentrations, ATP and 5-hydroxytryptamine had the same effect. When collagen fibres are directly exposed, as in the defective artery with platelet thrombus at 17 days, depicted in Fig 14, the connective tissue factor may be important in the initiation of the thrombosis.

On the whole, the development of the platelet thrombus through the stages of apparent fusion and ballooning was the same as in the platelet plug (*Jorgensen & Borchgrevink* 1963 a and b). The ballooning of the platelets did not reach the same degree as in some of the human platelet plugs, but here interspecies differences must be considered. The morphological development of the platelet plugs in rabbits were slower than in man. Nevertheless, the thrombotic aggregates had certain features in common with human plugs formed under defective coagulation (*Jorgensen & Borchgrevink* 1964). There was obviously a prolonged phase of fragmentation, embolization, and rebuilding of many thrombi. The reason for this may be that in the swift flow platelet aggregates are immediately torn away before enough thrombin has had time to form on the platelet surface to stabilize the aggregate.

Fragmentation, embolization, and rebuilding of fresh platelet thrombi have been described by all *in vivo* observers of thrombosis, first by *Jones* (1851). However, most of these studies have been based on short time experiments, lasting hours at the most. In the present investigation evidence is presented that in rabbits this may go on for several weeks. A clinical counterpart in man is probably the repeated transient attacks of monocular visual loss caused by platelet emboli in the retinal vessels in cases of mural carotid thrombosis (*Russell* 1961, *McBrien*,

Bradley & Ashton 1963) Situations analogous to this may well be common even in other vascular beds

Even though the precipitation and further development of platelet thrombi are dependent on humoral factors as ADP and thrombin the significance of haemodynamic factors must not be underrated (Eberth & Schummelbusch 1886 a Aschoff 1913 Murphy Rowsell Downie Robinson & Mustard 1962) They are probably decisive for the exact localization of the platelet thrombi Otherwise their particular occurrence at sites of eddying could not be explained Rowsell Geissinger & Mustard (1964) found platelet aggregation especially corresponding to sites of changed flow pattern when a diffuse endothelial injury was produced In our experiments platelet massing was preferentially located at and downstream to the stenosis in accordance with the experience from flow chambers (Shionoya 1957 Mustard Murphy Rowsell & Downie 1962) and vascular grafts (Eiken 1961) Further the regular spacing of the platelet columns and the coralline structure can hardly be accounted for without ascribing a great importance to the presence of eddies The increasing volume of coralline aggregates towards the open lumen may be due to increasing turbulence of flow as the thrombus is encroaching the lumen

Two circumstances should predispose to a more rapid accumulation of platelets in arteries than in veins More platelets per unit of time are carried to the site of thrombosis and turbulence is more likely to arise at a local restriction of the lumen due to the more rapid flow On the other hand there is no doubt that platelet thrombosis is hampered by the crumbling effect of the swift arterial flow In the present experiments there was a certain but not statistically significant trend for arterial platelet thrombi to grow more slowly than venous However at 2 days and later large aggregates prevailed in the non intentional arterial thrombi Less than half of these at 1-8 days was occluding while this was the case in all non intentional venous thrombi by the addition of a coagulation part This of course causes a break in the further growth of the original platelet thrombus The delay in the precipitation of the coagulation part in many non intentional arterial thrombi gave the platelet thrombus time to reach a considerable size Thus the often stressed factor of retarded blood flow appears to be of minor importance to platelet thrombi *per se* when the local stimulus to their formation is strong and enduring

On the other hand in the genesis of coagulation thrombi hindrance to the blood flow plays a major role (Zahn 1875 Eberth & Schummelbusch 1886 c) It has been considered unlikely that thrombin could form via the intrinsic clotting system unless there is almost complete stagnation of the blood (French 1962) Wessler (1955) produced coagulation thrombi at occluding ligatures by infusion of serum containing a clot promoting substance while ligation or serum infusion alone did not have this effect However already Baumgarten (1886) maintained

In spite of this, the initial step in the toxin-produced thrombosis in flowing blood was not fibrin precipitation, but platelet adhesion and aggregation. In accordance with the electronmicroscopical findings of *Poole, French & Cliff* (1963) the earliest platelet thrombus consisted of loose aggregates of granular platelets. They were morphologically identical to the platelet plugs in wounds where the clotting was most impaired (*Jorgensen & Borchgrevink* 1964). The same type of aggregate was produced *in vitro* by the addition of ADP to platelet-rich plasma, confirming the electronmicroscopical observations by *Hovig* (1962). Likewise in conformity with *Hovig* (1962), aggregates formed by tendon "extract" were, by the same time, generally more dense. But the density varied, which gave the masses a clumpy appearance, unlike most platelet aggregates in thrombosis or haemostasis. These findings are compatible with the concept that it is ADP, and not connective tissue, which brings about the initial aggregation. If so, it is also likely that the initial adhesion of the platelets to the damaged wall is precipitated by ADP, as previously indicated in the case of human haemostasis (*Jorgensen & Borchgrevink* 1964). *Honour & Mitchell* (1963) demonstrated *in vivo* that application of ADP to the vascular wall prompted platelet thrombosis but, in higher concentrations, ATP and 5-hydroxytryptamine had the same effect. When collagen fibres are directly exposed, as in the defective artery with platelet thrombus at 17 days, depicted in Fig 14, the connective tissue factor may be important in the initiation of the thrombosis.

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Platelets were found in connection with coagulation thrombi in 3 situations (1) as knots in the delicate net (2) as larger aggregates adhering to the coarse membranes (3) as secondary platelet thrombi on the free surface. Already at  $\frac{1}{2}$  hour the knots of the net were larger than those found in the recently formed *in vitro* clots and in the haematoma of the test wounds shortly after the bleeding (*Jorgensen & Borchgrevink 1963 a*). According to *Benthaus & Grunberg (1958)* this is due to retraction of the fibrin net. The larger aggregates were similar to those produced *in vitro* by thrombin except that some of the former had slightly degranulated platelets not matched in the latter. However in the electron microscope thrombin induced aggregates do show a varying degree of degranulation (*Hovig 1962*) although the platelets are not swollen and their cytoplasm not rarified to such a degree that one should expect to recognize the changes as ballooning in the light microscope. The close contact of the larger aggregates with the fibrin membranes makes it likely that even they are formed in the clotting milieu at the surface between two laminae of flow. Secondary platelet thrombi on the free surface of coagulation thrombi have been known since *Aschoff (1892)* and they may be explained by the great affinity of platelets to fibrin (*Schimmelbusch 1885*) and exudation of clot promoting serum from the retracting coagulation thrombus (*Quick 1950*).

As expected the amount of fibrin in the thrombi increased with time often up to a point where any original structure was concealed by hyaline masses of fibrin. The platelet thrombi underwent fibrinous transformation similar to that found in the platelet plug (*Jorgensen & Borchgrevink 1963 a*) but the intermediate stage of shrunken platelets and thick perimetric membrane was persistent in several instances especially in the middle of mixed thrombi. This and the fact that the coarse fibrin masses were particularly found at the upstream end of the thrombi point to the importance of an infiltration of plasma into the thrombus. The role played by local retraction of the clotting is probably illustrated by the tendency of the fibrin to precipitate in association with any collection of platelets—or at the damaged wall at the injection site in the primary coagulation thrombi. The poor effect of heparin in preventing the increase of fibrin may partly be a consequence of the neutralizing action of platelet factor 4.

The observation of fibrinous transformation of platelet aggregates may form a bridge between the two schools of adherents to the encrustation theory in the genesis of atherosclerosis viz those who consider fibrin precipitation as the primary event (*Duguid 1946 Crawford & Lutene 1957 Astrup 1959*) and those who stress the importance of platelet thrombi in this connection (*Hand & Chandler 1962 Murphy Rousell Downie Robinson & Mustard 1962*).

The increase of fibrin was succeeded by fibrinolysis both in a general and local form. The morphological criteria of general fibrinolysis

that complete arrest of flow is not a prerequisite for the formation of a coagulation thrombus, and Wessler (1955) showed that only partial ligation combined with serum infusion gave coagulation thrombi, although less frequently. Shionoya (1927) observed directly in his extra-corporeal shunt that the clotting started while blood still was flowing. In the present study complete occlusion of the vessels was not effected by platelet thrombi alone, but by precipitation of a coagulation part, more readily in veins than in arteries when the issue was not complicated by the stenosing ligature. The coagulation thrombi had a framework of fibrin membranes often running in streamline patterns, and they were the first structures to be laid down. Most likely, these membranes are formed at the contact surface between two laminae of flow, conceivably promoted by exchange of, and a steady new supply of activated clotting factors at this level. The frequent connection of the membranes with the platelet thrombi suggests release of clotting activity from these structures. In the coagulation thrombi upstream to the occluding ligature the presence of the coarse fibrin framework is consistent with a slow eddying movement of blood during their development and growth. Similar fibrin membranes, appearing as ring figures, were observed by Jorgensen & Borchgrevink (1964) in the haematoma of test wounds in patients with prolonged bleeding time, whereas they were not found in clots formed in completely non-moving blood (Jorgensen 1964 b). Here only a delicate net was observed, similar to that which was secondarily precipitated within the framework of the thrombi. All this evidence supports the concept that coagulation thrombi are formed in retarded, but still flowing blood, and that the occurrence of well-defined fibrin membranes in a coagulation thrombus is a strong indication of its intravital origin. These membranes were mentioned by Zahn (1875) and Aschoff (1892), but later authors have usually found that coagulation thrombi do not differ from clots (Welch 1910, Wessler, Reimer, Freiman, Reimer & Lertzman 1959, French 1962).

After this, one should expect a primary coagulation thrombus to form when the vessel is treated with staphylococcal toxin and partial ligation, creating both hypercoagulability and retardation of flow. When this did not occur, it may be explained by the fact that platelet adhesion and aggregation takes place more rapidly than intravascular clotting. Admittedly, in some instances the toxin and the ligature may have been of greater importance to the coagulation part than the modest amount of platelets which had gathered before the clotting. However, it may be difficult to reconstruct the part played by platelet massing in the formation of the individual mixed thrombus from only one observation. As shown by Baumgartner, Studer & Reber (1963) profound structural changes may occur during the establishment of a mixed thrombus, perhaps resulting in a less conspicuous permanent platelet part than what should be expected from the size of the original platelet thrombus.

Platelets were found in connection with coagulation thrombi in 3 situations (1) as knots in the delicate net (2) as larger aggregates adhering to the coarse membranes (3) as secondary platelet thrombi on the free surface. Already at  $\frac{1}{2}$  hour the knots of the net were larger than those found in the recently formed *in vitro* clots and in the haematoma of the test wounds shortly after the bleeding (*Jorgensen & Borchgrevink 1963 a*). According to *Benthaus & Grunberg (1958)* this is due to retraction of the fibrin net. The larger aggregates were similar to those produced *in vitro* by thrombin except that some of the former had slightly degranulated platelets not matched in the latter. However in the electron microscope thrombin induced aggregates do show a varying degree of degranulation (*Hovig 1962*) although the platelets are not swollen and their cytoplasm not rarified to such a degree that one should expect to recognize the changes as ballooning in the light microscope. The close contact of the larger aggregates with the fibrin membranes makes it likely that even they are formed in the clotting milieu at the surface between two laminae of flow. Secondary platelet thrombi on the free surface of coagulation thrombi have been known since *Aschoff (1892)* and they may be explained by the great affinity of platelets to fibrin (*Schimmelbusch 1885*) and exudation of clot promoting serum from the retracting coagulation thrombus (*Quick 1950*).

As expected the amount of fibrin in the thrombi increased with time often up to a point where any original structure was concealed by hyaline masses of fibrin. The platelet thrombi underwent fibrinous transformation similar to that found in the platelet plug (*Jorgensen & Borchgrevink 1963 a*) but the intermediate stage of shrunken platelets and thick perimetric membrane was persistent in several instances especially in the middle of mixed thrombi. This and the fact that the coarse fibrin masses were particularly found at the upstream end of the thrombi point to the importance of an infiltration of plasma into the thrombus. The role played by local activation of the clotting is probably illustrated by the tendency of the fibrin to precipitate in association with its collection of platelets—or at the damaged wall at the injection site in the primary coagulation thrombi. The poor effect of heparin in preventing the increase of fibrin may partly be a consequence of the neutralizing action of platelet factor 4.

The observation of fibrinous transformation of platelet aggregates in its form a bridge between the two schools of adherents to the encrustation theory in the genesis of atherosclerosis viz. those who consider fibrin precipitation as the primary event (*Duguid 1946 Crawford & Levine 1952 Astrup 1959*) and those who stress the importance of platelet thrombi in this connection (*Hand & Chandler 1962 Murphy Rowseell Downie Robinson & Mustard 1967*).

The increase of fibrin was succeeded by fibrinolysis both in a general and local form. The morphological criteria of general fibrinolysis



have been studied by *Sandritter & Bergerhof* (1954 a and b) and *Sandritter, Bergerhof & Kroker* (1954). The lysis entailed splitting and disappearance of fibrin bands, and the upstream end of the thrombus was first attacked. The platelet part of mixed thrombi was more resistant to lysis than the coagulation part. These findings are confirmed in the present study. It should be added that fragmentation of the peripheral membrane and disappearance of the delicate net allowed a marked escape of erythrocytes, which seemed to be the main cause of the reduction and fissuration of older thrombi. When platelet aggregates hidden by fibrin were attacked, the platelets were unmasked, apparently unaffected by the lysis. *Wolf* (1961) showed *in vitro* that platelet masses were microscopically unaltered by plasmin digestion, but their mechanical strength was poor.

The fibrinolysis did not usually become manifest before the 5th–7th day, earlier and more marked in the thromboplastin-made—and venous—thrombi, than in the toxin-produced—and arterial—thrombi. The relative resistance of the toxin-produced thrombi agrees with *Freiman, Bang & Clifton* (1960) who found that thrombi formed by chemical injury to the vascular wall were less amenable to fibrinolytic therapy than coagulation thrombi. If staphylokinase was present in the toxin preparation, it does not appear to have played any rôle. The greater fibrinolysis in veins may be related to the larger fibrinolytic potency of the venous wall (*Todd* 1958). However, it may also be accounted for by the rich venous collateral circulation through tributaries, lacking in arteries (*Clifton* 1963). Actually, the fact that the dissolution started at the upstream end of the thrombi, is more in favour of an effect of factors carried with the blood than by local tissue activators.

Heparin treatment both hastened and increased the fibrinolysis, in accordance with *Rabinovitch & Pines* (1943), *Loewe, Hirsch & Grayzel* (1947), *Sandritter & Bergerhof* (1954 a), and *Sandritter, Bergerhof & Kroker* (1954). *Wright, Kubik & Hayden* (1952) got the same result by treatment with a prothrombin depressant and ascribed this to a relative preponderance of the fibrinolysis when clotting is impaired. Considering the slight effect of heparin on the increase of fibrin, this cannot account for the present observations. More likely is a direct stimulation of fibrinolysis by heparin (*Halse* 1947, *von Kaulla & McDonald* 1958). The reduced number of toxin-produced thrombi under heparin treatment may be due to the preventive effect of heparin on the thrombus still not established at the initiation of the therapy. The number of the more readily formed thromboplastin-made thrombi was not reduced. Thus, the increased fibrinolysis did not definitely result in complete disappearance of the fully developed thrombi. However, this may be related to the presence of ligatures which prevented embolization of partly lysed material.

Many of the freely floating platelet aggregates observed in vessels with restored circulation may be completely detached thrombi origin-

ally formed on the vascular lining for platelets may adhere and clump even on apparently normal endothelium (Samuels & Webster 1952 Geissinger Mustard & Rowsell 1962) The mentioned aggregates went through the usual development from a loose collection to a dense granular mass However the most advanced of them had an appearance which did not fit in with an originally adherent platelet thrombus They had a granular centre surrounded by a faint fibrin membrane and only on the outside of this ballooned platelets occurred Aggregates identical to these were produced *in vitro* by tissue thromboplastin and recalcification plus ADP Further, they had great resemblance to the *in vitro* thrombi formed in the moving column of blood in the circulating loop described by Chandler (1958) (Poole 1959 French & Poole 1963 Chandler 1964) This favours the concept that the freely floating aggregates in the rabbit vessels were formed in moving blood independent of the wall and point to the importance of an

They may be explained if we assume  
the formation of a dense aggregate  
of platelets Jorgensen & Borch

grevink (1964) proposed that the ballooning is produced by thrombin on the platelet surface at the height of the thrombin effect When clotting is initiated dense platelet aggregates is conceivably formed as soon as trace amounts of thrombin appears while the clotting on the surface does not become sufficiently stimulated for the creation of ballooning Secondly this takes place only on the surface of the peripheral platelets In platelet plugs and thrombi the coagulation on the platelet surface is perhaps more strongly triggered as the platelets are caught one by one but even in the thrombi the ballooning was more marked on the outside of the fibrin membrane than on the inside

The particular trend for the freely floating aggregates to form under heparin treatment probably reflects the platelet clumping effect of this substance (Copley 1948 Ashwin 1961 Mustard, Murphy, Downie & Rowsell 1963 Nordoy & Odgaard 1963)

## SUMMARY AND CONCLUSIONS

Arterial and venous thrombi were produced in rabbits by two methods injection of staphylococcal toxin into the vascular wall combined with partial ligation and injection of tissue thromboplastin into a temporarily isolated section of the vessel One fourth of the animals received heparin treatment after the initiation of the thrombosis The rabbits were sacrificed at varying intervals from  $\frac{1}{2}$  hour to 50 days after the injection and the histological changes of the thrombi were followed until they became organized The observations were compared with rabbit platelet plugs and platelet aggregates formed in citrated platelet rich plasma by ADP connective tissue, tissue thromboplastin recalcification and thrombin

have been studied by *Sandritter & Bergerhof* (1954 a and b) and *Sandritter, Bergerhof & Kroker* (1954). The lysis entailed splitting and disappearance of fibrin bands, and the upstream end of the thrombus was first attacked. The platelet part of mixed thrombi was more resistant to lysis than the coagulation part. These findings are confirmed in the present study. It should be added that fragmentation of the peripheral membrane and disappearance of the delicate net allowed a marked escape of erythrocytes, which seemed to be the main cause of the reduction and fissuration of older thrombi. When platelet aggregates hidden by fibrin were attacked, the platelets were unmasked, apparently unaffected by the lysis. *Wolf* (1961) showed *in vitro* that platelet masses were microscopically unaltered by plasmin digestion, but their mechanical strength was poor.

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Staphylococcal toxin gave platelet or mixed thrombi, tissue thromboplastin coagulation thrombi. The platelet thrombi were located to sites of eddying and underwent the same changes as platelet plugs, usually ending in complete fibrinous transformation of the aggregates. Mural thrombi tended to break up and embolize over several weeks. Complete occlusion was effected by formation of a coagulation thrombus. The coagulation thrombi contained a framework of fibrin membranes running in streamline pattern. Platelets were connected with the coagulation thrombi as knots in the fibrin net, as larger aggregates adhering to the membranes, or as secondarily formed platelet thrombi on the free surface. There was a steady increase of fibrin, particularly in association with any aggregate of platelets. Fibrinolysis started at the upstream end of the thrombi after 5-7 days in non-heparinized animals, after 2-3 days in the heparinized ones. The fibrinolysis was more marked in the latter group, while the heparin treatment had only slight influence on the increase of fibrin.

The morphological development of platelet plugs in rabbits was slower than in man. Aggregates formed by ADP were small and loose, and the platelets granular. Connective tissue gave larger, unevenly dense aggregates. Aggregates induced by coagulation were dense and granular, tissue thromboplastin and recalcification gave a fringe of ballooned platelets. The same picture was found in freely floating platelet aggregates in vessels with restored circulation, particularly in the heparinized animals.

The following *conclusions* are drawn

The observations in the earliest stage of platelet thrombosis are compatible with the concept that in the present experiments ADP, and not connective tissue, brings about the initial adhesion and aggregation.

The localization and modelling of the platelet thrombi are dependent on haemodynamic factors, particularly the formation of eddies, while retardation of the flow is of minor importance when the local stimulus to platelet thrombosis is strong and enduring.

Mural platelet thrombi tend to break up and embolize over an extended period of time.

Coagulation thrombi are formed in retarded, but still flowing blood, and their membranes are probably formed at the contact surface between two laminae of flow.

Both the increase of fibrin and the fibrinolysis are probably related to plasma infiltration into the thrombus. In the former case the local activation of the clotting is probably associated with the platelets, in the latter case local tissue activators probably play a minor role compared with factors carried with the blood.

The increased fibrinolysis under heparin treatment is probably caused by direct stimulation of fibrinolysis and is not secondary to impaired clotting.

The freely floating platelet aggregates are probably formed by clotting in flowing blood independent of the wall, as in Chandler's loop

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## ACTION OF PARAINFLUENZA VIRUS TYPE 3 ON SYNTHESIS OF INTERFERON AND MULTIPLICATION OF HETEROLOGOUS VIRUSES

By

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Received 01 ix 64

Mixed viral infections often lead to one of the viruses inhibiting the multiplication of the other. This type of viral interaction has been associated with inhibitors of virus multiplication, interferon substances which are produced by cells exposed to virus (for references see 19) foreign nucleic acids (27) or nucleic acid derivatives (21).

It has also been shown that a virus can stimulate the multiplication of another virus (16, 20, 23). The stimulating effect of parainfluenza virus type 3 (PIV-3) on the multiplication of Newcastle disease virus (NDV) seems to be due to PIV-3 inhibiting the production and antiviral action of interferon (16). The effect of PIV 3 on production of interferon and multiplication of heterologous viruses has been further analysed in the present study.

### MATERIAL AND METHODS

**Cell cultures.** Primary cultures of pig and calf cells were prepared from trypsinized kidney tissue. The cells were grown at 37° C. in Hanks' salt solution supplemented with 0.5 per cent lactalbumin hydrolysate (Hanks + LAH), 10 per cent calf serum and antibiotics (100 I.U. penicillin and 100 µg streptomycin/ml). The same medium without serum or Eagle's minimal essential medium (MEM) (11) were used for maintenance. Cultures of HeLa cells were prepared as described previously (17).

**Viruses.** The PIV 3 strains used had been isolated from cattle or human being. The bovine strains were strain 23 (10), strain SF 4 (26), strain V 325 62 (2), strain R 24 (3), strains H 219 Sd 2 and Fc isolated by Dr K Bogel, strain T 1 (8) and strain Brookes isolated by Dr P S Dawson. All these strains had been isolated and transferred in several passages in calf kidney cultures. The human strains were strain I 4102 (6) and strain 222. The latter strain was supplied by Dr F R Abinanti and had been isolated in monkey kidney cells and was then transferred in 3 passages in calf cells in this laboratory. Strain FA102 had been isolated in KB cells and had undergone 5 passages in calf kidney cultures. Seed virus suspensions of the PIV-3 strains consisted of culture fluid harvested 3 days after infection of calf kidney cultures with virus at an input multiplicity (im) of about 0.01.

The author is indebted to Dr F R Abinanti, Bethesda, Dr F Burk, Bern, Dr K Bogel, Tübingen, Dr C Chang, Paris and Dr P S Dawson, Weybridge, who kindly supplied different PIV-3 strains. A gift of actinomycin D from Dr L Philipson, Uppsala, is gratefully acknowledged.

Challenge infections in interference experiments or interferon assays were carried out with strain Sendai of paramyxovirus type 1, strain 11 of pseudorabies virus, strain 80 61 of bovine enterovirus, strain 15 of swine influenza virus and strain F206 of poliovirus type 1. Stock suspensions of Sendai virus, bovine enterovirus and swine influenza virus were prepared in calf kidney cultures and pseudorabies virus was prepared in pig kidney cultures. Infectious RNA from poliovirus strain F206 was prepared as described previously (18). The PIV-3 strains were stored at 4° C and the other viruses at -60° C.

**Virus titrations.** Infectivity was measured as described earlier (17) by plaque assay in HeLa cells or by endpoint titration in roller tube cultures of calf kidney cells. In some experiments infectivity of PIV 3 was assayed in tube cultures inoculated with 0.2 ml of fourfold dilutions of the samples into groups of 10 cultures. The titre was then computed by the most probable number method (5).

In mixed viral infections, PIV 3 was neutralized by addition of PIV-3 antiserum as described previously (16).

Assay of haemagglutinin (HA) was carried out as described previously (17).

**Assay of interferon.** Interferon activity was assayed by two different methods. Viral activity was removed by dialysis at pH 2.0, ultracentrifugation and addition of antiviral serum as described previously (17). Twofold dilutions of the samples were inoculated into groups of 2 roller tube cultures. The cultures were incubated at 37° C for 18 to 24 hours and were subsequently challenged with 10<sup>4</sup> TCD<sub>50</sub> of Sendai virus. After incubation for another 3 to 4 days the media were assayed for haemagglutinin and the titre expressed as the reciprocal of the highest dilution which reduced the HA yield by 75 per cent.

When a more sensitive method - maintenance medium were inoculated and grown in plastic petri dishes. After washed in phosphate buffered salt forming units (PFU) of poliovirus room temperature the cultures were added. After incubation for 24 h -60° C. The media were subsequently assayed for poliovirus infectivity by plaque titration. The interferon titre was calculated on the basis of the observed linear relationship between log reduction of poliovirus yield and log concentration of interferon (18). Since it has not been ascertained whether different interferon preparations give the same regression coefficient the interferon titre was calculated from dilutions which reduced the poliovirus yield 50 to 95 per cent and from a regression of -0.81 the mean for 9 different titrations. The interferon titre was expressed in units representing the reciprocal of the dilution of the sample which reduced the poliovirus yield by 50 per cent.

**Irradiation (19).** The virus suspension was centrifuged at 15 000 g for 60 minutes and the pellet resuspended in 0.15 M NaCl buffered with 0.05 M Tris.

## RESULTS

### *Production of Interferon in Calf Cells Infected with Different PIV 3 Strains*

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV-3. It was possible, however, that such cultures produced small amounts of interferon which might have escaped detection. The production of interferon was therefore studied by a more sensitive assay method (18) than that used previously.

Flask cultures of calf cells were infected with strain 23 (in ~ 0.1), incubated at 37° C and assayed for interferon activity by challenge with

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*Virus titrations.* Infectivity was measured as described earlier (17) by plaque assay in HeLa cells or by endpoint titration in roller tube cultures of calf kidney cells. In some experiments infectivity of PIV 3 was assayed in tube cultures in

Assay of haemagglutinin (HA) was carried out as described previously (17).

*Assay of interferon.* Interferon activity was assayed by two different methods

After incubation for another 3 to 4 days the media were assayed for haemagglutinin and the titre expressed as the reciprocal of the highest dilution which reduced the HA yield by 75 per cent.

When a more sensitive method was required fourfold dilutions of the samples in maintenance medium were inoculated into groups of 1 to 2 calf kidney cultures.

After 48 hr the media were subsequently assayed for poliovirus infectivity by plaque titration. The interferon titre was calculated on the basis of the observed linear relationship between log reduction of poliovirus yield and log concentration of interferon (18). Since it has not been ascertained whether different interferon preparations give the same regression coefficient the interferon titre was calculated from dilutions which reduced the poliovirus yield 50 to 95 per cent and from a regression of 0.81 the mean for 9 different titrations. The interferon titre was expressed in units representing the reciprocal of the dilution of the sample which reduced the poliovirus yield by 50 per cent.

## RESULTS

### *Production of Interferon in Calf Cells Infected with Different PIV 3 Strains*

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV 3. It was possible, however, that such cultures produced small amounts of interferon which might have escaped detection. The production of interferon was therefore studied by a more sensitive assay method (18) than that used previously.

Flask cultures of calf cells were infected with strain 23 (im ~ 0.1), incubated at 37° C and assayed for interferon activity by challenge with

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## ACTION OF PARAINFLUENZA VIRUS TYPE 3 ON SYNTHESIS OF INTERFERON AND MULTIPLICATION OF HETEROLOGOUS VIRUSES

By

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Received 31 v 64

Mixed viral infections often lead to one of the viruses inhibiting the multiplication of the other. This type of viral interaction has been associated with inhibitors of virus multiplication, interferon substances which are produced by cells exposed to virus (for references see 19) foreign nucleic acids (27) or nucleic acid derivatives (21).

It has also been shown that a virus can stimulate the multiplication of another virus (16, 20, 23). The stimulating effect of parainfluenza virus type 3 (PIV-3) on the multiplication of Newcastle disease virus (NDV) seems to be due to PIV-3 inhibiting the production and antiviral action of interferon (16). The effect of PIV-3 on production of interferon and multiplication of heterologous viruses has been further analysed in the present study.

### MATERIAL AND METHODS

**Cell cultures.** Primary cultures of pig and calf cells were prepared from trypsinized kidney tissue. The cells were grown at 37° C. in Hanks' salt solution supplemented with 0.5 per cent lactalbumin hydrolysate (Hanks + LAH), 10 per cent calf serum and antibiotics (100 I.U. penicillin and 100 µg streptomycin/ml). The same medium without serum or Eagle's minimal essential medium (MEM) (11) were used for maintenance. Cultures of HeLa cells were prepared as described previously (17).

**Viruses.** The PIV-3 strains used had been isolated from cattle or human. *Leine* strain R 21 (3), strains R 219, Sd 2 and I 6 isolated by Dr K. Hogel, strain T 1 (8) and strain Brookes isolated by Dr P. S. Dawson. All these strains had been isolated and transferred in several passages in calf kidney cultures. The "human" strains were strain FA102 (6) and strain 2225. The latter strain was supplied by Dr F. R. Abinanti and had been isolated in monkey kidney cells and was then transferred in 3 passages in calf cells in this laboratory. Strain FA102 had been isolated in KB cells and had undergone 5 passages in calf kidney cultures. Seed virus suspensions of the PIV-3 strains consisted of culture fluid harvested 3 days after infection of calf kidney cultures with virus at an input multiplicity (im) of about 0.01.

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Challenge infections in interference experiments or interferon assays were carried out with strain Sendai of parainfluenza virus type 1 strain III of pseudorabies virus strain 80 61 of bovine enterovirus strain 15 of swine influenza virus and strain E206 of poliovirus type 1. Stock suspensions of Sendai virus, bovine enterovirus and swine influenza virus were prepared in calf kidney cultures and pseudorabies virus was prepared in pig kidney cultures. Infectious RNA from poliovirus strain E206 was prepared as described previously (18). The PIV-3 strains were stored at 4° C and the other viruses at -60° C.

**Virus titrations.** Infectivity was measured as described earlier (17) by plaque assay in HeLa cells or by endpoint titration in roller tube cultures of calf kidney cells. In some experiments infectivity of PIV 3 was assayed in tube cultures inoculated with 0.2 ml of fourfold dilutions of the samples into groups of 10 cultures. The titre was then computed by the most probable number method (5).

In mixed viral infections PIV 3 was neutralized by addition of PIV 3 antiserum as described previously (16).

Assay of haemagglutinin (HA) was carried out as described previously (17).

**Assay of interferon.** Interferon activity was assayed by two different methods.

After incubation for another 3 to 4 days the media were assayed for haemagglutinin and the titre expressed as the reciprocal of the highest dilution which reduced the HA yield by 75 per cent.

When a medium was maintained in plaque forming unit room temperature added after

-60° C. The media were subsequently assayed for poliovirus infectivity by plaque titration. The interferon titre was calculated on the basis of the observed linear relationship between log reduction of poliovirus yield and log concentration of interferon (18). Since it has not been ascertained whether different interferon preparations give the same regression coefficient the interferon titre was calculated from dilutions which reduced the poliovirus yield 50 to 95 per cent and from a regression of -0.81 the mean for 9 different titrations. The interferon titre was expressed in units representing the reciprocal of the dilution of the sample which reduced the poliovirus yield by 50 per cent.

**Ultraviolet (UV) irradiation.** The virus suspension was centrifuged at 10 000 g for 60 minutes and the pellet resuspended in 0.15 M NaCl buffered with 0.02 M Tris-HCl.

## RESULTS

### *Production of Interferon in Calf Cells Infected with Different PIV 3 Strains*

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV 3. It was possible however that such cultures produced small amounts of interferon which might have escaped detection. The production of interferon was therefore studied by a more sensitive assay method (18) than that used previously.

Flask cultures of calf cells were infected with strain 23 (in  $10^{-1}$ ), incubated at 37° C and assayed for interferon activity by challenge with

poliovirus RNA No interferon activity was demonstrated in the medium, when tested in a dilution of 1:4 as shown in Table 1

Other strains of PIV-3 were examined similarly for their ability to induce production of interferon in calf cells. The results of the interferon assays are given in Table 2. High titres of interferon were found in cultures infected with the strains 2225 and LA102. No or only slight interferon activity was demonstrated in cultures infected with strains SI 4, V 325/62, Brookes, E6, R-2A, R-2V9 and Sd-2, although more haemagglutinin was produced in these cultures than in cultures infected with the strains 2225 and LA102. An interferon activity of 200 units was demonstrated in the medium of cultures infected with strain T 1.

TABLE 1  
*Absence of Interferon Activity in Medium of Calf Kidney Cultures Infected with Strain 23 of PIV-3*

| Period of incubation in days | Yield of poliovirus in per cent of control |      | Estimated titre on interferon |
|------------------------------|--|------|-------------------------------|
|                              | Dilution of culture medium tested          |      |                               |
|                              | 1:1  | 1:16 |                               |
| 2                            | 100  | 140  | <4                            |
| 4                            | 126  | 172  | <4                            |
| 6                            | 67   | 100  | <4                            |

TABLE 2  
*Yield of Interferon in Calf Kidney Cultures Infected with Different Strains of PIV-3*

| PIV 3 strain | Yield of poliovirus in per cent of control |      |      |       |        |        |        |        |         | Estimated titre of interferon |
|--------------|--|------|------|-------|--------|--------|--------|--------|---------|-------------------------------|
|              | Dilution tested                            |      |      |       |        |        |        |        |         |                               |
|              | 1:4  | 1:16 | 1:64 | 1:256 | 1:1000 | 1:1024 | 1:2000 | 1:8000 | 1:10000 |                               |
| SI 4         | 77   | 100  | 110  | 144   | -      | 125    | -      | -      | -       | <4                            |
| V 325/62     | 42   | 96   | 124  | 88    | -      | 110    | -      | -      | -       | 5                             |
| T 1          | -  | 60   | 42   | 84    | -      | 58     | -      | -      | -       | 200                           |
| Brookes      | 81   | -    | 118  | 160   | -      | 120    | -      | -      | -       | <4                            |
| E 6          | 87   | 100  | 78   | -     | -      | 91     | -      | -      | -       | <4                            |
| R 2A         | 104  | 100  | 115  | -     | -      | 109    | -      | -      | -       | <4                            |
| R 2V9        | 41   | 84   | 123  | -     | -      | 111    | -      | -      | -       | 5                             |
| Sd 2         | 83   | 96   | 110  | -     | -      | 125    | -      | -      | -       | <4                            |
| LA102        | -  | -    | -    | -     | 18     | -      | 16     | 31     | 88      | 10 000                        |
| 2225         | -  | 0.56 | 2.9  | 13    | -      | 39     | -      | -      | -       | 1 000                         |

- = not done

#### *Influence of Environmental Conditions on Production of Interferon*

The influence of temperature on interferon production in PIV-3 infected cultures was studied since Rutz-Gomez & Isaacs (28) have shown an increased yield of interferon at temperatures above the optimal temperature for virus growth.

Calf kidney cultures were infected with strain 23 (im ~ 10), and in-

cubated at different temperatures. The medium was assayed for haemagglutinin daily and for interferon after incubation for 4 days. Interferon was assayed by challenge with poliovirus RNA. The production of haemagglutinin was not markedly influenced by variations of the temperature between 33° and 38.5° C, but was inhibited significantly by incubation at 40° C. Interferon activity, however, was not demonstrated in cultures incubated at 40° C or in cultures incubated at the lower temperatures (Table 3).

TABLE 3

*Influence of Different Conditions on Production of Interferon in Cultures Inoculated with Strain 23 of PIV 3*

| Cultures assayed for interferon                      | Yield of poliovirus in per cent of control |      |      |       |        |        | Estimated titre of interferon |
|--|--|------|------|-------|--------|--------|-------------------------------|
|  | Dilution tested                            |      |      |       |        |        |                               |
|  | 1:4  | 1:16 | 1:64 | 1:256 | 1:1024 | 1:4096 |                               |
| PIV 3 infected calf cultures                         |  |      |      |       |        |        |                               |
| incubated at 33°                                     | 72   | 96   | 179  | 103   | —      | —      | <4                            |
| incubated at 37°                                     | 67   | 119  | 99   | 96    | —      | —      | <4                            |
| incubated at 38.5°                                   | 63   | 72   | —    | —     | —      | —      | <4                            |
| incubated at 40°                                     | 90   | 73   | 140  | 88    | —      | —      | <4                            |
| with acid medium pH 6.8                              | 55   | 96   | —    | —     | —      | —      | <4                            |
| PIV-3 infected pig cultures                          |  | 45   | 18   | 39    | 142    | —      | 300                           |
| Calf cultures inoculated with heat inactivated PIV 3 | 26   | 69   | 10   | 58    | —      | —      | 300                           |
| UV irradiated PIV-3                                  | —  | —    | 0.82 | 4.6   | 7.6    | 53     | 10 000                        |

— not done

Slightly acid conditions are known to increase the yield of interferon in rat cells infected with Sindbis virus (9). A similar experiment was therefore carried out with calf kidney cultures infected with strain 23 (im ~ 10). The pH of culture fluids were adjusted to either 6.8 or 7.4 by addition of CO<sub>2</sub>. No interferon, however, was demonstrated in infected cultures incubated with acid medium for 3 days (Table 3).

Whether or not infection with strain 23 could induce interferon synthesis in cells other than calf cells was also studied. Pig cells were chosen since these cells produce interferon which can be assayed in calf cells (24). Flask cultures of pig cells were infected with strain 23 (im ~ 10) and incubated for 3 days at 37° C. The medium of these cultures and uninfected controls were subsequently assayed for interferon. No interferon was found in uninfected cultures when the medium was tested in a dilution of 1:16 by challenge with poliovirus RNA. The infected cultures, on the other hand, had an interferon activity of 300 units (Table 3).

#### *Interferon Production by Inactivated Virus*

Samples of the stock suspension of strain 23 were incubated at 37° C for 48 hours or irradiated with UV-light for 2 minutes. These treatments reduced the infectivity 3 and 4 log units respectively. The virus sus-



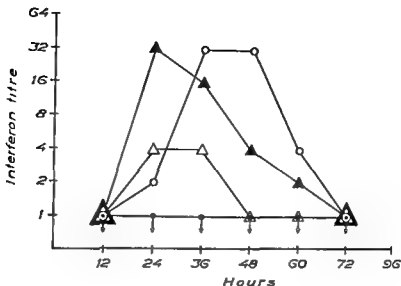


Fig 1.

Production of interferon in calf kidney cultures inoculated with UV irradiated and untreated virus of two different PIV 3 strains

- ▲—▲ Untreated virus EA102
- Untreated virus 23
- UV-irradiated virus 23
- △—△ UV-irradiated virus EA102

pensions were inoculated in 2 ml amounts into calf kidney cultures in plastic petri dishes. The cultures were incubated at 37° C for 1 hour, washed and maintenance medium added. The culture fluids were then assayed for interferon by challenge with poliovirus RNA after 3 days at 37° C. Interferon activity was found both in cultures inoculated with heat-inactivated virus and in cultures inoculated with UV-irradiated virus (Table 3). The highest concentration of interferon was found in cultures exposed to UV-irradiated virus. The interferon titre in these cultures was of the same magnitude as in cultures infected with strain EA102 (cf Table 2).

The rate at which interferon was produced in calf cells treated with the strains 23 and EA102 was studied by exposing stock suspensions to UV light for 2 minutes as described in Material and Methods. Untreated and irradiated virus was then inoculated into separate plate cultures of calf cells. The cultures were washed twice after 1 hour at 37° C and fresh medium added. The medium was changed every 12 hours and assayed for interferon activity by challenge with Sendu virus.

The results in Fig 1 show that interferon was produced more rapidly in cultures inoculated with untreated virus of strain EA102 than in cultures inoculated with UV-irradiated virus of strain 23. Maximum yields of interferon were obtained after incubation for 24 hours with strain EA102 and for 36 hours with strain 23. Less interferon was obtained with the strain EA102 after UV-irradiation of virus.

The influence of UV light on virus 23 was further analyzed by irradiation for various periods. After UV-irradiation the virus suspensions

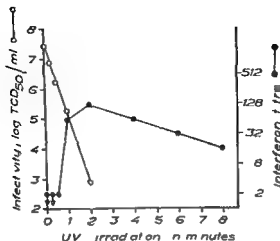


Fig 2

The effect of UV irradiation on infectivity of strain 23 and on interferon production in calf kidney cultures inoculated with this virus

were diluted tenfold in maintenance medium assayed for infectivity and inoculated in 2 ml amounts into plate cultures of calf cells. The cultures were washed twice after 1 hour at 37° C and supplied with fresh medium. The culture fluids were subsequently assayed for interferon by challenge with Sendai virus after further incubation of the cultures for 2 days at 37° C.

The decrease of the infectivity per minute of irradiation was found to be 2.2 log units (Fig 2). Maximum interferon titre was obtained with virus irradiated for 2 minutes. The yield of interferon decreased gradually after further irradiation and was 1/8 the maximum yield after irradiation for 8 minutes.

The possibility was examined whether the interferon production obtained with UV irradiated strain 23 might be due to infection with an interferon inducing virus, e.g. a UV resistant mutant. Strain 23 was UV irradiated for 2 minutes and inoculated into a culture of calf cells. The medium of this culture was harvested after 2 days at 37° C and inoculated in serial tenfold dilutions into roller tube cultures of calf cells. These cultures were incubated for 3 days at 37° C after which the cells were examined for cytopathic changes and the medium for interferon by challenge with Sendai virus. The infectivity of the medium from the culture inoculated with UV irradiated virus was found to be  $10^{1.5}$  TCD<sub>50</sub> per ml. No interferon was demonstrated in cultures inoculated with this medium in dilutions  $10^1$  to  $10^4$  thus indicating that the interferon production obtained with UV irradiated virus 23 was not induced by infectious virus.

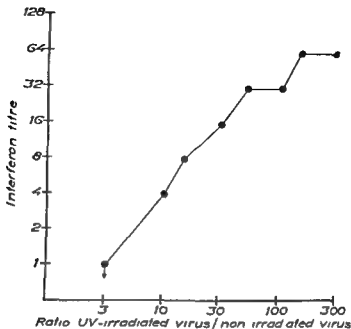


Fig 3

The inhibitory effect of infectious virus on interferon production by UV irradiated virus of strain 23

#### *Effect of Non-Irradiated Virus on Interferon Production by UV-Irradiated Virus*

The influence of strain 23 on interferon production was investigated in an experiment in which different dilutions of untreated virus were added to equal volumes of virus UV-irradiated for 2 minutes. Calf kidney cultures were inoculated with these suspensions and assayed for interferon after incubation for 2 days at 37° C. The yield of interferon was found to decrease at increasing concentrations of non-irradiated virus (Fig 3). No reduction of the interferon yield was demonstrated when the UV-irradiated virus contained less than 1 per cent untreated virus. With 10 per cent untreated virus the interferon yield was reduced 16 times and with 30 per cent untreated virus more than 64 times.

#### *Inactivation of Interferon in PIV 3 Infected Cultures*

It was shown in a previous communication (16) that an infection with strain 23 of PIV-3 inhibits production and antiviral action of interferon in calf cells. This effect might be caused by an inactivation of interferon in PIV 3 infected cultures.

To study this hypothesis flask cultures of calf kidney cells were infected with strain 23 ( $m \sim 10$ ). After incubation for 3 days at 37° C the medium was concentrated about tenfold by dialysis against powder of polyethylene glycol 20 M. The cells were removed from the glass, sedimented by centrifugation and resuspended in 0.15 M NaCl buffered to pH 7.3 with 0.02 M Tris-buffer. This cell suspension which contained

about  $5 \times 10^7$  cells per ml was disintegrated in a Potter Elvehjem grinder. The concentrated medium and the disintegrated cells in volumes of 1.5 ml were then mixed with 0.5 ml NDV interferon (17) or 0.5 ml Hanks + LAH. The suspensions were incubated at 37° C for 24 hours and assayed for interferon by challenge with Sendai virus. To test if intact cells could destroy interferon, cells from infected cultures were overlaid with 5 ml interferon or 0.5 ml Hanks + LAH, incubated at 37° C for 24 hours and assayed for residual interferon activity in the fluid phase. Materials from uninfected cultures were treated in the same way.

No significant reduction of the interferon titre was obtained when interferon was incubated with intact cells, disintegrated cells or concentrated medium from PIV-3 infected cultures (Table 4). This result does not support the hypothesis that interferon is inactivated in calf kidney cultures infected with strain 23 of PIV-3.

TABLE 4

*Failure to Demonstrate Inactivation of Interferon by Incubation of NDV Interferon with Medium, Disintegrated and Intact Cells from Calf Kidney Cultures Infected with Strain 23 of PIV-3*

| Incubation          | Titre of Interferon     |               |                     |               |
|---------------------|-------------------------|---------------|---------------------|---------------|
|                     | PIV-3 infected cultures |               | Uninfected cultures |               |
|                     | Interferon              | No interferon | Interferon          | No interferon |
| Medium              | 8                       | <2            | 4                   | <2            |
| Disintegrated cells | 8                       | <2            | 8                   | <2            |
| Intact cells        | 8                       | <2            | 8                   | <2            |

### *Presence of Incomplete Virus in PIV-3 Infected Cultures*

Synthesis of interferon in L cells infected with NDV appears to be induced by incomplete virus (18). If this is also valid for calf cells infected with PIV-3 it could be expected that smaller amounts of incomplete virus are produced in cells infected with strain 23 than in cells infected with strain I A102 since an infection with the latter virus gave rise to more interferon.

Calf kidney cultures were infected with these PIV-3 strains (im ~ 0.1) and incubated at 37° C. The medium was changed every 12 hours and assayed for infectivity and haemagglutinin. Strain I A102 was assayed for infectivity in HeLa cells and strain 23 in roller tube cultures of calf cells. The infectivity was expressed in plaque forming units (PFU) for strain I A102 and in most probable number of cytopathic units (MPNCU) for strain 23. The HA titrations were performed both at 4° C and at 37° C. Table 5 shows that the ratio between the number of infectious and haemagglutinating units was not significantly higher in cultures infected with strain 23 than in cultures infected with strain I A102. The mean of this ratio was approximately  $5 \times 10^{-3}$  when haem

agglutinin was assayed at 4° C and approximately  $0.3 \times 10^6$  when the assay was performed at 37° C. Thus noninfectious, haemagglutinating virus does not appear to be produced in high concentration in calf kidney cultures infected with strain EA102.

TABLE 5  
Yield of Haemagglutinin and Infectious Virus in Cultures Infected with PIV-3 Strains EA 102 and 23

| Incubation in hours | HA units/ml   |           | PFU/ml $\times 10^{-4}$ | MPNCL/ml $\times 10^{-4}$ | Ratio infectivity HA $\times 10^{-4}$ |            |
|---------------------|---------------|-----------|-------------------------|---------------------------|---------------------------------------|------------|
|                     | Strain EA 102 | Strain 23 | Strain EA 102           | Strain 23                 | Strain EA 102                         | Strain 23  |
| 12                  | <4 (<4)       | <4 (<4)   | —                       | —                         | —                                     | —          |
| 24                  | <4 (4)        | 4 (64)    | 1.5                     | 23                        | — (0.38)                              | 5.8 (0.36) |
| 36                  | 8 (128)       | 16 (512)  | 63                      | 235                       | 7.9 (0.49)                            | 15 (0.46)  |
| 48                  | 4 (128)       | 32 (512)  | 37                      | 90                        | 9.3 (0.29)                            | 2.8 (0.19) |
| 60                  | 4 (64)        | 32 (1024) | 13                      | 191                       | 3.3 (0.20)                            | 6.0 (0.19) |
| 72                  | <4 (32)       | 32 (1024) | 11                      | 441                       | — (0.34)                              | 14 (0.43)  |

Figures in brackets refer to HA titres obtained at 37°

— = not done

#### *Interference between PIV-3 and Some Heterologous Viruses*

Interference between PIV-3 and heterologous viruses was studied in calf kidney cultures infected with either the strain EA102 or the strain 23 (im ~ 10). The PIV-3 infected cultures and uninfected controls were incubated at 37° C for 1 day and superinfected with  $10^4$  to  $10^7$  TCD<sub>50</sub> of pseudorabies virus, bovine enterovirus, swine influenza virus or Sendai virus. After 2 hours at 37° C the infected cultures were washed twice and supplied with fresh medium. Samples of the culture fluids were withdrawn at intervals and assayed for infectivity after neutralization of PIV-3 by antiserum.

Fig. 4 shows that a previous infection with strain EA102 strongly inhibited the multiplication of all viruses tested, whereas strain 23 only inhibited Sendai virus. Strain 23 appeared on the other hand to promote the multiplication of pseudorabies virus. Evidence for a stimulation of the multiplication of pseudorabies virus was also obtained when the cultures were assayed for pseudorabies infectivity after freezing and thawing.

#### *The Action of Strain 23 on the Multiplication of Poliovirus in Calf Cells*

To ensure that the observed interference between strain 23 of PIV-3 and parainfluenza virus type 1 was not due to presence of interferon in the cells, the PIV-3 infected cultures were challenged with poliovirus RNA. Such an infection has previously been found to be extremely sensitive to interferon (18). Calf kidney cells grown in plastic petri dishes were infected with strain 23 (im ~ 10) and incubated for 1 day

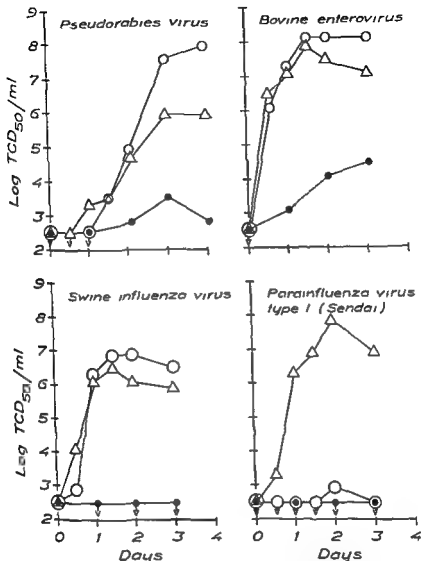


Fig 4

The multiplication of some challenge viruses in calf kidney cultures previously infected with strain 23 (○—○) or strain F4102 of PIV 3 (●—●) Control cultures infected with challenge virus only (Δ—Δ)

at 37° C. The PIV-3 infected cultures and uninfected controls were then inoculated with 80 PFU of poliovirus RNA according to the technique described in Material and Methods. The cultures were incubated at 37° C for 36 hours and assayed for poliovirus infectivity. No suppression of the poliovirus occurred in cultures previously infected with PIV 3 (Table 6). This mixed infection yielded 6 times higher titres of

poliovirus in the medium than the single infection with poliovirus RNA. Similar results were obtained when the cultures were assayed after freezing and thawing. Nor was it possible to detect higher concentrations of poliovirus in the cell phase than in the medium after treatment of the cells with 8 M urea for 30 seconds at room temperature.

TABLE 6  
*Yield of Poliovirus in PIV-3 Infected Cultures after Infection with 80 PFU of Poliovirus RNA*

| Culture        | Yield of poliovirus in PFU per culture |                |                    |
|----------------|--|----------------|--------------------|
|                | Medium                                 | Cells + Medium | Urea treated cells |
| PIV-3 infected | 5 600                                  | 7 300          | 1 400              |
| Control        | 880                                    | 1 000          | 640                |

The increased yield of poliovirus in cultures infected with strain 23 might be ascribed to the fact that this virus inhibits the production of interferon (16). It was therefore examined whether an increased multiplication of poliovirus also occurred in cultures treated with actinomycin D, since this substance is considered to inhibit the synthesis of interferon (12, 14) but not multiplication of small RNA viruses (25). The effect of actinomycin on interferon synthesis and on a DNA virus was investigated in cultures infected with NDV ( $\text{im} \sim 10$ ) and pseudorabies virus ( $\text{im} \sim 5$ ), respectively. After infection the cultures were incubated for 1 day at 37° C and assayed for infectivity, haemagglutinin and interferon as shown in Table 7.

TABLE 7

*The Influence of Strain 23 of PIV 3 and Actinomycin Respectively on the Multiplication of Poliovirus in Calf Kidney Cultures Infected with Poliovirus RNA*

| Concentration of actinomycin $\mu\text{g/ml}$ | Poliovirus PFU/ml $10^{-3}$ |         | Pseudorabies virus $\log \text{TCID}_{50}/\text{ml}$ |         | NDV HA units/ml |         | Interferon Units 5 ml $\times 10^{-3}$ |
|---|-----------------------------|---------|--|---------|-----------------|---------|--|
|   | PIV 3 infected              | Control | PIV 3 infected                                       | Control | PIV 3 infected  | Control | NDV infected                           |
| 0.10  | 12                          | 0.52    | 3.5  | 3.9     | 64              | 64      | 0.8                                    |
| 0.05  | 9.3                         | 1.6     | 4.7  | 5.7     | 64              | 64      | 2                                      |
| 0.025   | 17                          | 2.8     | 6.7  | 7.1     |                 |         | -                                      |
| 0.000   | 18                          | 3.5     | 8.1  | 7.9     | 32              | 32      | 10                                     |

The multiplication of poliovirus was found to be inhibited in cultures treated with actinomycin in concentrations exceeding 0.025  $\mu\text{g}$  per ml medium. In the presence of 0.1  $\mu\text{g}$  per ml the poliovirus yield was reduced to 15 per cent of that of controls inoculated with poliovirus RNA only. The production of interferon in NDV infected cultures was also significantly decreased at this concentration of actinomycin although the multiplication of NDV did not appear to be inhibited. An increased

yield of poliovirus was found in actinomycin treated cultures which prior to the inoculation of poliovirus RNA and actinomycin had been infected with strain 23 (Table 7). It was also found that the inhibitory effect of actinomycin on pseudorabies virus was not diminished in cultures previously infected with strain 23.

## DISCUSSION

In a previous investigation (16) no interferon could be demonstrated in calf kidney cultures infected with strain 23 of PIV-3. Neither was interferon demonstrated in such cultures by a more sensitive method for interferon assay (18) as shown in this study. The PIV-3 infected cells were also susceptible to infection with poliovirus RNA which has been found to be sensitive to interferon (18). Furthermore interferon was not demonstrated in PIV-3 infected cultures under conditions which increase the production of interferon in other cell virus system—incubation in slightly acid medium (9) or at temperatures supra optimal for virus growth (28). These results taken together thus indicate that strain 23 can multiply in calf cells without inducing synthesis of interferon.

The production of interferon was also studied in calf cells infected with other PIV-3 strains. No or only slight interferon activity was obtained with 7 of 8 bovine strains whereas 2 strains of human origin gave rise to high titres of interferon. Although no interferon was found in calf cultures infected with the bovine strain 23, interferon was produced by this virus in pig cultures. These results support the hypothesis (28) that production of interferon by a virus is not an intrinsic property of the virus particles but a property associated with a particular cell virus interaction. The findings also indicate that less interferon is produced by a virus infection when the virus is well adapted to the cells. It is of interest in this connection that interferon synthesis is induced by heterologous nucleic acids but not by homologous (27).

An increased production of interferon was obtained in calf kidney cultures inoculated with strain 23 after heat inactivation or UV irradiation of virus. Similar results have previously been obtained with other viruses (1, 4, 13, 22). The results obtained with strain 23 indicate that UV irradiation destroys the part of the viral genome responsible for suppression of interferon synthesis. Results which supported this hypothesis were (a) interferon production in cultures inoculated with UV irradiated virus did not appear to be induced by infectious virus, (b) interferon activity was not destroyed in cultures infected with strain 23, (c) non irradiated virus inhibited production of interferon by UV irradiated virus and (d) an infection with strain 23 inhibited inter-



infectivity. An evaluation of this question is complicated by the fact that infectious virus even in low concentration inhibited interferon production by UV-irradiated virus, a phenomenon previously described by Lindenmann for another cell-virus system (22).

It could not be shown that interferon production in calf kidney cultures infected with the human strain LA102 was due to formation of non-infectious, haemagglutinating virus. The ratio infectivity/haemagglutinin was approximately  $8 \times 10^6$  when the HA assay was performed at  $4^\circ \text{C}$ . The same ratio was obtained for strain 23. Furthermore, less interferon was produced by UV-irradiated virus of strain LA102 than by non-irradiated virus. These results therefore indicate that the difference in interferon-inducing capacity between the strains 23 and LA102 might be due to different ability of the viruses to inhibit production of interferon.

Although no interferon was found in calf kidney cultures infected with strain 23, such cultures showed a high resistance against infection with Sendai virus. No interference was found, on the other hand, between strain 23 and such unrelated viruses as bovine enterovirus, pseudorabies virus, swine influenza virus and poliovirus. Since Sendai virus is less sensitive to the antiviral action of interferon in calf cells than poliovirus RNA (18) these results indicate that interference occurs between heterologous but related viruses in the absence of interferon. Similar findings have been shown by Crowell & Syverton in another cell virus system (7).

Furthermore, an infection with strain 23 stimulated the multiplication of poliovirus in calf cells infected with poliovirus RNA. Since the strain 23 can stimulate the multiplication of heterologous virus by inhibiting the production and antiviral action of interferon (16) the enhanced multiplication of poliovirus might be due to this effect. However, the yield of poliovirus was decreased in cultures treated with actinomycin, although this compound inhibited production of interferon. An infection with strain 23 also enhanced the multiplication of poliovirus in actinomycin-treated cultures. It is therefore possible that strain 23 stimulates the multiplication of poliovirus by another mechanism than by inhibition of interferon production.

#### SUMMARY

No or only slight interferon activity was found in calf kidney cultures infected with some bovine strains of parainfluenza virus type 3 (PIV-3), whereas high titres of interferon were obtained by infections with PIV-3 strains of human origin. An increased production of interferon was obtained with the bovine strain 23 after UV-irradiation of virus, but not with the human strain LA102. The production of interferon in cultures treated with UV irradiated virus was inhibited by presence of

non irradiated virus of strain 23 This inhibition increased at higher concentrations of untreated virus

Different types of interaction between PIV-3 and heterologous viruses are described Interference mediated by interferon seems to occur between strain EA102 and unrelated viruses as pseudorabies virus and bovine enterovirus, interference without interferon between strain 23 and Sendai virus, and stimulation of virus multiplication in cultures concurrently infected with strain 23 and poliovirus RNA The stimulating effect of strain 23 on the multiplication of poliovirus in calf cells does not appear to be due to an inhibited production of interferon

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No or only slight interferon activity was found in calf kidney cultures infected with some bovine strains of parainfluenza virus type 3 (PIV-3), whereas high titres of interferon were obtained by infections with PIV-3 strains of human origin. An increased production of interferon was obtained with the bovine strain 23 after UV-irradiation of virus, but not with the human strain EA102. The production of interferon in cultures treated with UV-irradiated virus was inhibited by presence of

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## STUDIES ON TRANSFORMATION IN MORAXELLA AND ORGANISMS ASSUMED TO BE RELATED TO MORAXELLA

2 Quantitative Transformation Reactions between *Moraxella*  
*nonliquefaciens* Strains, with Streptomycin Resistance Marked DNA

By

KJELL BOVRE

Received 21 III 64

The relationships in terms of transformation between strains of the same species, classified according to conventional criteria, are of interest also for the following reasons

- 1 For comparative transformation studies it is necessary to have strains which are representative of their species
- 2 Information concerning the level and general distribution of interstrain transformation rates within a species is necessary for the estimation of a first degree interstrain relationship in terms of transformation. An intraspecies transformation rate unit of this kind is fundamental for the evaluation of transformation ratios for eventual classification purposes
- 3 As a consequence of homogeneity or heterogeneity in terms of transformation the taxonomic significance of minor phenotypic deviations may be better understood

A relatively broad study of the assumed species is preferable for the elucidation of these aspects. Previous reports on heterologous transformation reactions in different groups of bacteria partly suffer from the lack of evidence that the strains applied are representative for the species in question. It may also be found that the descriptions of the strains used in such studies are insufficient. A basis of specified conventional diagnostic criteria is, in the opinion of the author, indispensable for the eventual use of transformation in taxonomy. In the absence of adequate information of this kind, the genetic findings will have questionable validity and few practical consequences. The use of type culture collection strains without proper control may be misleading (see below)

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regularly 15-20  $\mu\text{g}$  per ml of the reaction mixture. Controls for DNA saturation consisted of parallels with 30-40  $\mu\text{g}$  DNA per ml which never gave means of transformant scores significantly deviating from those at the lower DNA concentration except for some cases with prolonged DNA exposure.

In attempts to transform the two mentioned recipients with DNAs from the strains 19116/51 and 752/52 there were also included experiments with continuous DNA exposure (without DNase treatment) during the expression period. In continuous DNA exposure DNA is acting in fluid medium for maximum 30 min. Then aliquots are plated and allowed to reach the stage of pin point colonies before exposure to streptomycin. There is no further DNA addition during incubation of the plates. As even an approximate quantitation under these conditions is most difficult no attempt has yet been made towards the exact fixation of transformation ratios based on continuous DNA exposure. However transformation of *Moraxella non liquefaciens* 7784 with DNA from a strain of *Moraxella bovis* has shown that continuous DNA exposure gives more than a hundredfold of transformants in relation to the initial number of recipient bacteria as compared with 30 min DNA exposure. This estimation has been made with the usual initial DNA concentration 20  $\mu\text{g}$  per ml.

The strains 19116/51 and 752/52 were also applied as recipients in transformation experiments using their own mutant DNA and DNA from other strains as transforming principle. In these experiments the same transformation procedure was applied although neither of the two strains had been studied in connection with the methodological analysis (Boore 1964).

Transformants were regularly scored at the concentrations 500  $\mu\text{g}$  streptomycin per ml of strain 7784 with DNA from and 10  $\mu\text{g}$  streptomycin per ml were experiment intrastrain transformants in 50, 30 and 10  $\mu\text{g}$  streptomycin per ml with the addition

replica plating  
formity of high

penicillin resistance in some of the donor strains

## RESULTS

### Description of the Strains

Microscopically the strains revealed plump rods, often very short diplococci occasionally occurring in long chains. The strain 752/52 was not as plump as the other strains. There was often a considerable variation in size and shape in the same microscopical preparation. Giant and filamentous forms were not seldom found in the young cultures. This feature was more pronounced in older cultures.

As a rule the bacteria seemed to divide in one plane. In some few of the preparations however there were infrequent occurrences of groups of 4 organisms which might perhaps have arisen by division in two opposite planes. All strains were Gram negative. The general impression was some resistance to decolorization. Irregular staining with strongly stained and pale cells was observed in some preparations. The morphological features of some of the strains are presented in Figs. 1-3.

The colonies on blood agar were of small or moderate size, regularly a little larger than 1 mm in diameter after 20 h incubation. They were round hemispherical or almost flat, often with a domed center and a flat periphery. There was no pigmentation. All variations between

## MATERIAL AND METHODS

*Moraxella nonliquefaciens* has been chosen as the starting material of these studies, because of its frequent occurrence in human material (Henriksen 1958 Kaffk & Blodorn 1959 a o), and because this species seems to be relatively 'clearcut' from the diagnostic point of view.

21 strains of *Moraxella nonliquefaciens* isolated in Oslo from clinical human material were included. The bacteriological diagnosis had been made by different bacteriologists during routine examinations, and the diagnosis had been based on the genus description proposed by Henriksen (1952) with the addition of the following characters: No haemolysis, no liquefaction of serum and no serum requirement. In addition to these 21 strains, 1 strain was received from The National Collection of Type Cultures, London under the name of *Moraxella lacunata* 7784. It was shown that this strain neither liquefied coagulated bovine serum nor required serum for growth, so that it could be added to the material of *Moraxella nonliquefaciens* strains.

The strains had been stored in the lyophilized state at  $+5^{\circ}\text{C}$  and were kept in subculture on blood agar for long periods. They were examined in the following way, partly as a consequence of transformation results which revealed the need for additional biochemical reactions and cultural characteristics distinguishing between strains with deviating transformation ratios.

20 h old blood agar surface cultures, cultivated at  $32-33^{\circ}\text{C}$  in a humid atmosphere were used for microscopical examinations, characterization of colony consistency for testing the ability of being emulsified in physiological saline and for detection of

|                             |
|-----------------------------|
| 1 per cent aqueous solution |
| was tested microscopically  |
| h 0.4 per cent agar         |

Surface cultures on Hugh & Lefson's medium were made after the addition of 1 per cent agar to the medium described by Hugh & Lefson (1953). The medium was prepared with Bacto-Peptone. Acid production from glucose was tested on glucose acetate agar slants (with 1 per cent glucose, 24 per cent acetate and phenol red indicator). Utilization of citrate as the sole source of carbon was read in Koser's medium (Koser 1924 medium no. [2]). Liquefaction of serum was tested on 92 per cent bovine serum in broth coagulated at  $80^{\circ}\text{C}$  for 2 h. The test for hydrogen sulphide production was performed in a meat extract peptone agar medium with 0.1 per cent lead acetate. The medium for indol production consisted of 2 per cent peptone (Pepton aus Casein tryptisch verdaut Merck) and 0.2 per cent  $\text{Na}_2\text{HPO}_4$  in distilled water and the medium for nitrite production from nitrate consisted of 0.02 per cent nitrite free  $\text{KNO}_3$  and 0.5 per cent Bacto Peptone in distilled water. The urease test was performed according to Blake Christensen (1946) with a very heavy inoculum from young blood agar cultures.

Gelatin liquefaction was not tested because of difficulties in assessing growth in the conventional medium.

Readings of growth on Hugh & Lefson's medium and in citrate-glucose utilization, hydrogen sulphide production, indol production and urea decomposition were made after 4 days incubation. Readings of nitrite production were performed daily for 4 days and a distinct red colour reaction on at least one occasion with the conventional sulphanilic acid-alphanaphthylamine reagent was considered a positive result. Remaining nitrate in negative reactions was tested for with the same reagents after reduction by means of Zn powder. All incubations were undertaken at  $32-33^{\circ}\text{C}$  except for some growth experiments which were run in parallel at  $37^{\circ}\text{C}$ .

Antibiotic sensitivity tests were performed according to the method of Ericsson Hogman & Wielman (1954) further described by Ericsson (1960) and commented by Boire (1962).

Streptomycin sensitive wild type *Moraxella nonliquefaciens* 4113/52 and 7784 were exposed to DNA from streptomycin resistant mutants of each of the 22 strains all selected at 500  $\mu\text{g}$  streptomycin per ml. The methods of mutant selection, DNA extraction and quantitative transformation have been described previously (Boire 1964). In the latter communication the above mentioned recipients were both described in detail with regard to their behaviour in the transformation system. Regularity of results reflecting methodological stability should therefore be the best obtainable. As a rule, the DNA exposure was allowed to proceed for 15 min., with DNase treatment at the end of exposure. The concentration of DNA was re-

gularly 15-20  $\mu\text{g}$  per ml of the reaction mixture. Controls for DNA saturation consisted of parallels with 30-40  $\mu\text{g}$  DNA per ml which never gave means of transformant scores significantly deviating from those at the lower DNA concentration except in some cases with prolonged DNA exposure.

In attempts to transform the two mentioned recipients with DNAs from the strains 19116/51 and 752/52 there were also included experiments with continuous DNA exposure (without DNase treatment) during the expression period. In continuous DNA exposure DNA was acting in fluid medium for maximum 30 min. Then aliquots are plated and allowed to reach the stage of pin point colonies before

per ml

The strains 19116/51 and 752/52 were also applied as recipients in transformation experiments using their own mutant DNA and DNA from other strains as transforming principle. In these experiments the same transformation procedure was applied although neither of the two strains had been studied in connection with the methodological analysis (Boire 1961).

Transformants were regularly scored at the concentration 500  $\mu\text{g}$  streptomycin per ml. Transformation of strain 7784 with DNA from strain 19116/51 and 10  $\mu\text{g}$  streptomycin per ml were carried out. Intrastrain transformants in 0.5 and 10  $\mu\text{g}$  streptomycin per ml.

The same procedure was applied to the recipient strain 4661/62 with the addition of the selection concentration 1000  $\mu\text{g}$  of streptomycin per ml.

Transformants were controlled for resistance to streptomycin by replica plating. This method was also applied for the determination of the uniformity of high streptomycin resistance in some of the donor strains.

## RESULTS

### *Description of the Strains*

Microscopically, the strains revealed plump rods, often very short diplobacilli, occasionally occurring in long chains. The strain 752/52 was not as plump as the other strains. There was often a considerable variation in size and shape in the same microscopical preparation. Giant and filamentous forms were not seldom found in the young cultures. This feature was more pronounced in older cultures.

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TABLE 1  
Some Characteristics and the Origin of the Assumed Moraxella nonliquefaciens Strains

| Strain** | Microscopic picture  | Consistency of colonies | Agglutination in physiological saline | Growth on the surface of Hugh & Leifson's medium | Growth in citrate | Nitrate production | Lysoc activity | Origin                        |
|----------|----------------------|-------------------------|---------------------------------------|--|-------------------|--------------------|----------------|-------------------------------|
| 672/58   | Usual*               | Mucoid                  | —                                     | —  | —                 | +                  | —              | Sputum, ozarcna               |
| 2770/60  | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose, ozarcna                 |
| 151/62   | Usual                | Intermediate†           | (+)                                   | —  | —                 | +                  | —              | Nose                          |
| 1911/65  | Usual                | Soft                    | —                                     | +  | +                 | —                  | —              | Clinical material (TC London) |
| 7784     | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 4378/62  | Usual                | Mucoid                  | —                                     | —  | —                 | +                  | —              | Nose, ozarcna                 |
| 3828/60  | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 4235/62  | Usual                | Mucoid                  | —                                     | —  | —                 | +                  | —              | Nose                          |
| 836/61   | Usual                | Intermediate            | (+)                                   | —  | —                 | +                  | —              | Nose                          |
| 178/62   | Usual                | Mucoid                  | —                                     | —  | —                 | +                  | —              | Nose                          |
| 752/52   | Smaller diameter†    | Mucoid                  | —                                     | +  | —                 | +                  | —              | Nose                          |
| 270/60   | Usual                | Mucoid                  | —                                     | —  | —                 | +                  | —              | Clinical material             |
| 820/61   | Usual                | Soft                    | —                                     | —  | —                 | +                  | +              | Nose                          |
| 13015/62 | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 4620/62  | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 4007/62  | Mainly coccoid*      | Intermediate            | (+)                                   | —  | —                 | +                  | —              | Nose                          |
| 4865/62  | Long chains of cocci | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 13785/62 | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Nose                          |
| 13576/62 | Usual                | Intermediate            | (+)                                   | —  | —                 | +                  | —              | Nose                          |
| 5050/62  | Usual                | Friable                 | +                                     | —  | —                 | +                  | —              | Nose                          |
| 5058/62  | Usual                | Friable                 | +                                     | —  | —                 | +                  | —              | Nose                          |
| 1962/62  | Usual                | Soft                    | —                                     | —  | —                 | +                  | —              | Clinical material             |

\*\* Denominator of designation indicates year of isolation

\* Cells mainly as presented in Fig. 1 † See Fig. 3 ‡ See Fig. 2 § Intermediate between soft and friable

|| Nitrate apparently not attacked as shown by means of /n powder reduction

opaque and clear colonies could be observed. Most of the strains mainly yielded colonies with a moderately opaque center and an almost clear periphery. Some strains had domed, greyish white colonies, indistinguishable from the common appearance of *Neisseria catarrhalis* colonies. In addition, two of these strains (5050/62 and 5058/62) regularly had friable colonies, which could not be completely emulsified in physiological saline. Other strains had colonies intermediate between friable and soft, and some grew with mucoid colonies. Most strains, however, grew with soft colonies, easily emulsified in saline (Table 1). All strains were nonhaemolytic. The strain 752/52, however, gave a greenish discolorization of the blood agar on prolonged incubation.

The delicacy of growth of these organisms on blood agar was almost similar to the growth characteristics of enterococci on the same medium. The strains 7784 and 752/52 were slightly more slow-growing than the other strains. All strains grew at least as well at 32-33° C as at 37° C on blood agar aerobically, some strains with a slight preference for the lower temperature. In a dry atmosphere at 37° C growth of some strains was retarded as compared with growth in a humid atmosphere at the same temperature. In fluid media 32-33° C also seemed to be a slightly better growth temperature than 37° C. All strains failed to grow anaerobically on blood agar and in fluid media. In 0.4 per cent Brain Heart Infusion agar stab culture all strains grew fairly well down to 5 mm below the surface. All strains grew well on ascites agar and bovine serum. As a rule the strains were not able to grow on Hugh & Lefson's medium (Table 1). Growth in the medium for hydrogen sulphide production was observable with some difficulties in all strains. All strains grew feebly in the indol medium described and somewhat better in the medium for nitrite production.

The strains were all immotile and oxidase positive, and did not produce acid from glucose, liquefy serum, produce indol or hydrogen sulphide. They were all strongly sensitive to penicillin, streptomycin, chloramphenicol, oxytetracycline and erythromycin (Table 2). Other characteristics and the origin of the strains are collected in Table 1.

TABLE 2

Sensitivity to Antibiotics of the 22 Strains Classified as *Moraxella nonliquefaciens*

| Antibiotic      | Range of inhibition zones in mm | Approximate range of minimum inhibitory concentrations (m.i.c.)* |
|-----------------|---------------------------------|--|
| Penicillin      | 33-43                           | 0.07-0.004 I.U./ml   |
| Streptomycin    | 23-29                           | 1-0.08 µg/ml   |
| Chloramphenicol | 30-42                           | 0.5-0.03 µg/ml   |
| Oxytetracycline | 24-34                           | 0.6-0.02 µg/ml   |
| Erythromycin    | 29-40                           | 0.5-0.02 µg/ml   |

\* M.i.c. values calculated from zone diameters by means of regression equations for each antibiotic (Ferguson 1960).

TABLE 1  
Some Characteristics and the Origin of the Assumed *Moraxella nonliquefaciens* Strains

| Strain** | Microscopical picture | Consistency of colonies | Agglutination in physiological saline | Growth on the surface of fluid in 1% tellurite medium | Growth in citrate | Nitrate production | Urease activity | Origin            |
|----------|-----------------------|-------------------------|---------------------------------------|---|-------------------|--------------------|-----------------|-------------------|
| 672/58   | Usual*                | Mucoid                  | —                                     | —   | —                 | +                  | —               | Sputum ozacna     |
| 2770/60  | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose ozacna       |
| 159/62   | Usual                 | Intermediate†           | (+)                                   | —   | —                 | +                  | —               | Nose              |
| 19116/51 | Usual                 | Soft                    | —                                     | +   | +                 | —                  | —               | Clinical material |
| 7784     | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | NYC London        |
| 4378/62  | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 3828/60  | Usual                 | Mucoid                  | —                                     | —   | —                 | +                  | —               | Nose              |
| 4295/62  | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 836/61   | Usual                 | Intermediate            | —                                     | —   | —                 | +                  | —               | Nose              |
| 178/62   | Usual                 | Soft                    | (+)                                   | —   | —                 | +                  | —               | Nose              |
| 732/52   | Smaller diameter†     | Mucoid                  | —                                     | —   | —                 | +                  | —               | Nose              |
| 270/60   | Usual                 | Mucoid                  | —                                     | +   | —                 | +                  | +               | Clinical material |
| 826/61   | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 13015/62 | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 4626/62  | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 4667/62  | Mainly coccoid*       | Intermediate            | (+)                                   | —   | —                 | +                  | —               | Nose              |
| 4867/62  | Long chains of cocci  | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 13385/62 | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
| 13536/62 | Usual                 | Intermediate            | —                                     | —   | —                 | +                  | —               | Nose              |
| 5050/62  | Usual                 | Irregular               | (+)                                   | —   | —                 | +                  | —               | Nose              |
| 5058/62  | Usual                 | Irregular               | +                                     | —   | —                 | +                  | —               | Nose              |
| 1962/62  | Usual                 | Soft                    | —                                     | —   | —                 | +                  | —               | Nose              |
|          |                       |                         | —                                     | —   | —                 | +                  | —               | Clinical material |

\*\* Denominator of designation indicates year of isolation

\* Cells mainly as presented in Fig 1 + See Fig 3 † See Fig 2

† Nitrate apparently not attacked as shown by means of /n powder reduction

‡ Intermediate between soft and friable

opaque and clear colonies could be observed. Most of the strains mainly yielded colonies with a moderately opaque center and an almost clear periphery. Some strains had domed, greyish white colonies, indistinguishable from the common appearance of *Neisseria catarrhalis* colonies. In addition, two of these strains (5050/62 and 5058/62) regularly had friable colonies, which could not be completely emulsified in physiological saline. Other strains had colonies intermediate between friable and soft and some grew with mucoid colonies. Most strains, however, grew with soft colonies, easily emulsified in saline (Table 1). All strains were nonhemolytic. The strain 752/52, however, gave a greenish discolorization of the blood agar on prolonged incubation.

The delicacy of growth of these organisms on blood agar was almost similar to the growth characteristics of enterococci on the same medium. The strains 7784 and 752/52 were slightly more slow-growing than the other strains. All strains grew at least as well at 32–33° C as at 37° C on blood agar aerobically, some strains with a slight preference for the lower temperature. In a dry atmosphere at 37° C growth of some strains was retarded as compared with growth in a humid atmosphere at the same temperature. In fluid media 32–33° C also seemed to be a slightly better growth temperature than 37° C. All strains failed to grow anaerobically on blood agar and in fluid media. In 0.4 per cent Brain Heart Infusion agar stab culture all strains grew fairly well down to 5 mm below the surface. All strains grew well on ascites agar and bovine serum. As a rule the strains were not able to grow on Hugh & Lefson's medium (Table 1). Growth in the medium for hydrogen sulphide production was observable with some difficulties in all strains. All strains grew feebly in the indol medium described and somewhat better in the medium for nitrite production.

The strains were all immotile and oxidase positive, and did not produce acid from glucose, liquefy serum, produce indol or hydrogen sulphide. They were all strongly sensitive to penicillin, streptomycin, chloramphenicol, oxytetracycline and erythromycin (Table 2). Other characteristics and the origin of the strains are collected in Table 1.

TABLE 2  
Sensitivity to Antibiotics of the 22 Strains Classified as *Moraxella nonliquefaciens*

| Antibiotic       | Range of inhibition zones in mm | Approximate range of minimum inhibitory concentrations (m.i.c.) <sup>a</sup> |
|------------------|---------------------------------|--|
| Penicillin       | 33–43                           | 0.07–0.004 I.U./ml   |
| Streptomycin     | 23–29                           | 1–0.08 µg/ml   |
| Chl. ramphenicol | 30–33                           | 0.5–0.03 µg/ml   |
| Oxytetracycline  | 24–34                           | 0.6–0.02 µg/ml   |
| Erythromycin     | 29–40                           | 0.5–0.02 µg/ml   |

<sup>a</sup> M.i.c. values calculated from zone diameters by means of regression equations for each antibiotic (Friesen in 1960).

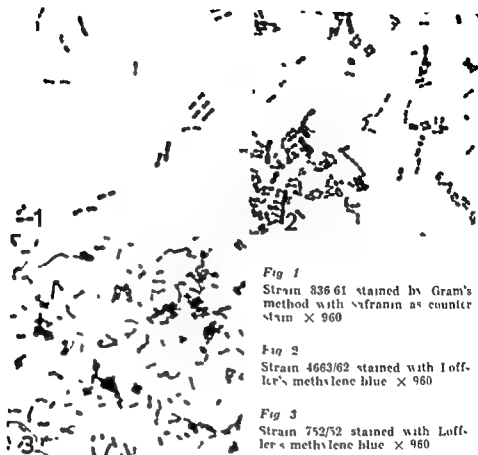


Fig 1

Strain 836/61 stained by Gram's method with safranin as counter stain  $\times 960$

Fig 2

Strain 4663/62 stained with Löffler's methylene blue  $\times 960$

Fig 3

Strain 752/52 stained with Löffler's methylene blue  $\times 960$

### *Transformation Reactions*

The transformation frequency for each donor—recipient combination was compared with simultaneously performed transformation of the recipient with its own mutant DNA, and the ratios of interstrain to intrastrain transformation were determined. The results are collected in Tables 3 and 4. It is evident that 20 out of the 22 strains show a rough uniformity as regards interstrain transformation, the ratios of inter- to intrastrain transformation ranging from  $3.4 \cdot 10^{-1}$  to  $9.9 \cdot 10^{-1}$ . Two strains are definitely deviating in terms of transformation, neither of their DNAs being able to transform members of the intertransformable group. That the DNA of the 19116/51 donor is in its active state, is shown in Table 5. The recipient strain 19116/51 is transformed by its own mutant DNA, but neither by DNAs from the intertransformable group nor from strain 752/52. The strain 752/52 was not transformable, and therefore the activity of its mutant DNA could not be sufficiently controlled. The physical and chemical condition of this DNA did not, however, distinguish itself from other DNAs as regards viscosity and concentration estimated in the Dische diphenylamine reaction.

In a separate experiment (not tabulated) a potent recipient culture

TABLE 3

*Ratios of Interstrain to Intrastrain Transformation to Streptomycin Resistance in Presumptive Moraxella nonliquefaciens Strains*  
*Recipient Moraxella nonliquefaciens 7784*

| Donor strain | Recipient count ml  | Interstrain transformants ml | Intrastrain transformants ml* | Ratio of inter- to intrastrain transformation |
|--------------|---------------------|------------------------------|-------------------------------|---|
| 672 58       | 1.4 10              | 1.3 10 <sup>5</sup> (127)†   | 2.4 10 <sup>5</sup> (242)     | 5.3 10 <sup>-1</sup>                          |
| 2770 60      | 6.0 10 <sup>6</sup> | 9.1 10 <sup>4</sup> (91)     | 1.4 10 <sup>5</sup> (143)     | 6.6 10 <sup>-1</sup>                          |
| 159 62       |                     |                              |                               | not tested                                    |
| 19116 51     | 1.5 10 <sup>8</sup> | < 10 <sup>1</sup> (0)‡       | 4.7 10 <sup>5</sup> (47)      | < 2.1 10 <sup>-3</sup>                        |
| 4378 62      | 1.1 10              | 7.9 10 <sup>4</sup> (79)     | 2.1 10 <sup>5</sup> (210)     | 3.8 10 <sup>-1</sup>                          |
| 3828 60      | 6.0 10 <sup>6</sup> | 9.0 10 <sup>4</sup> (90)     | 1.4 10 <sup>5</sup> (143)     | 6.3 10 <sup>-1</sup>                          |
| 4233/62      | 1.1 10              | 1.1 10 <sup>5</sup> (114)    | 2.1 10 <sup>5</sup> (210)     | 5.4 10 <sup>-1</sup>                          |
| 836 61       | 1.4 10 <sup>7</sup> | 1.1 10 <sup>5</sup> (105)    | 2.4 10 <sup>5</sup> (242)     | 4.3 10 <sup>-1</sup>                          |
| 178/62       | 6.0 10              | 8.4 10 <sup>4</sup> (84)     | 1.4 10 <sup>5</sup> (143)     | 5.9 10 <sup>-1</sup>                          |
| 752/62       | 1.5 10 <sup>8</sup> | < 10 <sup>1</sup> (0)‡       | 4.7 10 <sup>5</sup> (47)      | < 2.1 10 <sup>-3</sup>                        |
| 270/60       | 1.1 10 <sup>7</sup> | 9.5 10 <sup>4</sup> (95)     | 2.1 10 <sup>5</sup> (210)     | 4.8 10 <sup>-1</sup>                          |
| 826 61       | 6.0 10 <sup>6</sup> | 1.2 10 <sup>5</sup> (115)    | 1.4 10 <sup>5</sup> (143)     | 8.0 10 <sup>-1</sup>                          |
| 13015/62     | 1.1 10 <sup>7</sup> | 7.7 10 <sup>4</sup> (77)     | 2.1 10 <sup>5</sup> (210)     | 3.7 10 <sup>-1</sup>                          |
| 4626 62      | 1.1 10 <sup>7</sup> | 8.3 10 <sup>4</sup> (83)     | 2.1 10 <sup>5</sup> (210)     | 4.0 10 <sup>-1</sup>                          |
| 4663/62      | 1.1 10              | 8.3 10 <sup>4</sup> (83)     | 2.1 10 <sup>5</sup> (210)     | 4.0 10 <sup>-1</sup>                          |
| 4863/62      | 1.4 10 <sup>7</sup> | 1.7 10 <sup>5</sup> (172)    | 2.4 10 <sup>5</sup> (242)     | 7.1 10 <sup>-1</sup>                          |
| 13387/62     | 2.3 10 <sup>7</sup> | 2.0 10 <sup>5</sup> (204)    | 3.1 10 <sup>5</sup> (307)     | 6.6 10 <sup>-1</sup>                          |
| 13536 62     | 2.3 10 <sup>7</sup> | 3.0 10 <sup>5</sup> (303)    | 3.1 10 <sup>5</sup> (307)     | 9.9 10 <sup>-1</sup>                          |
| 5030 62      | 2.3 10 <sup>7</sup> | 1.6 10 <sup>5</sup> (159)    | 3.1 10 <sup>5</sup> (307)     | 5.2 10 <sup>-1</sup>                          |
| 5058 62      | 2.3 10              | 2.4 10 <sup>5</sup> (235)    | 3.1 10 <sup>5</sup> (307)     | 7.7 10 <sup>-1</sup>                          |
| 1962 62      | 6.0 10 <sup>6</sup> | 1.1 10 <sup>5</sup> (106)    | 1.4 10 <sup>5</sup> (143)     | 7.4 10 <sup>-1</sup>                          |

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

\* Intrastrain transformants have been scored in simultaneous transformation of the recipient with its own mutant DNA

† Figures in brackets indicate means of three (in a few cases two) plate counts

‡ In parallel continuous DNA exposure transformants were still not detected

of strain 7784 (30 min exposure to homologous DNA giving 3.2 10 transformants per ml at each of the transformant selection concentrations 50 and 10 µg streptomycin per ml) was exposed continuously to DNA from strain 75252. No interstrain transformants were scored at 10 or 10 µg streptomycin per ml verifying the results from transformation at the 500 µg selection concentration.

The other two supplementary experiments which were undertaken in intrastrain transformation of the strains 7784 and 4663 62 to discover events of heterogeneity or deviation of the degree of streptomycin resistance in transformants similarly did not reveal any differences in the counts of transformants with selection concentrations varying from 1000 to 10 µg streptomycin per ml.

On no occasion transformants were found which did not grow freely on the surface of blood agar with 1000 µg streptomycin per ml. All donor strains were able to grow at this concentration of streptomycin when simply streaked on the surface. Replica plating of 100 colonies of each of the donors 7784 and 4378 62 without exception yielded growth at 1000 µg streptomycin per ml.

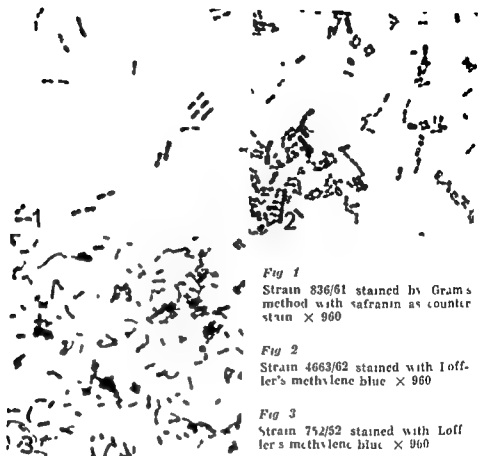


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Department of Bacteriology, Karolinska Institutet Stockholm and Department of Bacteriology, Serafimerlasarettet, Stockholm

## TRANSFORMATION OF STREPTOMYCIN-RESISTANCE IN *BORDETELLA PERTUSSIS*<sup>1</sup>

By

P BRANEFORS

Received 25 III 64

A change in the properties of a bacterial strain can sometimes be obtained by means of exposure of the bacteria to DNA from a related strain

During the last decade this technique, transformation, has become an important tool in microbial genetics *Diplococcus pneumoniae* (8, 9), *Haemophilus influenzae* (2, 3, 10), *Neisseria meningitidis* (6), and a few other species have been studied in this respect Pneumococci and *H. influenzae* are probably the best studied species so far A few years ago transformation was also found in conjunction with *Bacillus subtilis* (11, 12, 13), a species that has many well-defined "markers" and consequently promises to be a good system for further genetic investigations

The present paper reports transformation in *Bordetella pertussis*, a species that has not previously been studied from this point of view A streptomycin-sensitive strain of *B. pertussis* has been rendered streptomycin resistant by means of exposure to DNA from a resistant variant of the same strain

### MATERIAL AND METHODS

*Strains* *Bordetella pertussis* strain 441227 was used as recipient in most experiments It is a virulent (phase I) strain which is used at the State Bacteriological Laboratory Stockholm Sweden for vaccine production This strain is sensitive to streptomycin in concentrations over 1 µg per ml on solid media

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The author wishes to express her thanks to Dr G Heden for many discussions and for his great interest in the work She also wishes to thank Dr J Spizizen for his helpful advice concerning the experimental conditions

<sup>1</sup> The research reported here has been made possible through the support and sponsorship of the U.S. Department of the Army through its European Research Office Contract number DA 91 591 F-1 ( 94701 1058 59



*Moraxella nonliquefaciens* These observations are at least promising as regards the use of transformation in classification

## SUMMARY

22 bacterial strains, classified as *Moraxella nonliquefaciens*, were investigated, using conventional diagnostic criteria and quantitative streptomycin resistance transformation

20 strains behaved in transformation as closely related strains, with ratios of inter- to intrastrain transformation ranging from 1/3 to 1, approximately. Members of this group of strains are considered representative for *Moraxella nonliquefaciens* for the use in transformation studies extended to other bacteria in the taxonomic vicinity of this species

2 strains deviated definitely in terms of transformation. These strains both revealed distinct cultural and biochemical characteristics which usually are not recorded or paid special attention to in the diagnosis of *Moraxella nonliquefaciens*

The results are considered promising for the use of transformation as an aid in the classification of these bacteria

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The donor strain was a streptomycin resistant mutant of the same strain.

<sup>1</sup> In discussions and  
he Spizizen for his

supported by the U.S. Department of the Army through its European Research Office Contract number DA 91-591 F(C) 94701 10-5 59

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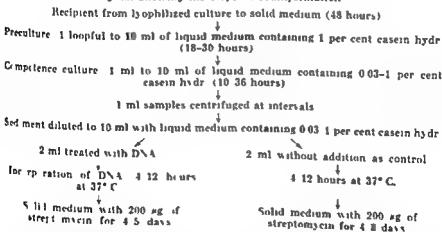
## EXPERIMENTAL

In the transformation experiments the liquid medium mentioned above was used with the omission of the casamino acids and  $\text{CuSO}_4$ . When performing the experiments a solution of vitamin free casein hydrolysate was added.

Strain 44122,7 was grown from a free dried culture at  $35^\circ\text{C}$  for 48 hours on the solid medium mentioned above. It gave a good confluent growth. The same agar slant could be used for about fourteen days if stored at  $4^\circ\text{C}$ . When performing an experiment one small loopful of bacteria was transferred to a 100 ml Erlenmeyer flask containing 10 ml of liquid medium to which 1 per cent casein hydrolysate had been added. It was incubated at  $37^\circ\text{C}$  on the rotary shaker until it had reached a density of about  $1 \times 10^9$  cells per ml. This took 18 to 30 hours. From this pre-culture 1 ml portions were transferred to a number of 100 ml flasks each containing 10 ml of liquid medium to which varying amounts of casein hydrolysate had been added from 0.03 per cent to 1 per cent. These cultures which were intended to develop competence for transformation were incubated on the rotary shaker for

The volume of the test samples usually was 2 ml. To each sample 0.1 ml of DNA was added diluted so that the final DNA concentration was approx.  $1 \mu\text{g}$  per ml sample. The samples in wide test tubes were placed at  $37^\circ\text{C}$  on the rotary shaker in order to permit cellular incorporation of DNA varying from 4 to 12 hours. Resistant mutants were screened for by transferring 0.1-0.5 ml of the cultures to the surface of the solid medium containing 200  $\mu\text{g}$  streptomycin per ml. The agar slants were incubated for 4 to 5 days at  $35^\circ\text{C}$ .

## Diagram Showing the Steps in Transformation



As mentioned above the negative controls consisted of 2 ml of the remaining centrifuged and diluted culture. Apart from the omission of the DNA these control samples were treated in the same manner as the test samples.

## RESULTS

Initially the transformation experiments were performed with the liquid medium that was later used only for DNA preparation. Under these experimental conditions resistant mutants were obtained only rarely.

An increase in the number of transformed bacteria was in some cases

|   |                 |    |
|---|-----------------|----|
| Bacto Casamino acids (Difco technical)            | 10              | g  |
| Yeast extract (Difco)                             | 25              | g  |
| Soluble starch (Merek)                            | 15              | g  |
| MgCl <sub>2</sub> 6 H <sub>2</sub> O 25% solution | 16              | ml |
| CaCl <sub>2</sub> 10% solution                    | 01              | ml |
| FeSO <sub>4</sub> 7 H <sub>2</sub> O 1% solution  | 10              | ml |
| CuSO <sub>4</sub> 5 H <sub>2</sub> O 1% solution  | 005             | ml |
| L-arginine 0.01% solution                         | 10              | ml |
| L-histidine 0.005% solution                       | 10              | ml |
| KH <sub>2</sub> PO <sub>4</sub>                   | 05              | g  |
|   | 1000 ml aq dest |    |

Ph 7.8

The agar slants were prepared by adding 15 per cent Bacto agar (Difco) to the liquid medium together with 3 per cent of a 7 per cent stock solution of starch. After sterilization 10 per cent human blood was added.

**Preparation of DNA** (modified from Spizizen (12)), strain 44122/7 R was grown on the solid medium for 48 hours at 35° C transferred to 500 ml of liquid medium in a 2 l Erlenmeyer flask, and grown on a rotary shaker (120 rpm stroke eccentricity 2.5 cm) at 37° C for 30 hours. The whole culture was then poured into a 6 l Erlenmeyer-flask containing 25 l of fresh medium and left on the rotary shaker for 48 to 60 hours. After this time the culture was in the stationary growth phase and had reached a concentration of at least  $1 \times 10^{10}$  bacteria per ml. Two to three such cultures were used for each preparation of DNA. The cells were centrifuged in an IEC serumcentrifuge for about 45 minutes (ca.  $2000 \times g$ ), and then washed with about 200 ml of 0.1 M NaCl containing 0.015 M Na citrate. This washing and the extractions and precipitations reported in the following were carried out at 4° C, except where otherwise stated. The bacterial suspension was centrifuged for 20 minutes in an IEC refrigerated centrifuge at  $3900 \times g$ . The washing and centrifuging of the cells was repeated once. The cells were then suspended in the smallest possible amount of 0.1 M NaCl containing 0.015 M Na citrate and transferred to a freeze press (Edebo (7)) for disintegrating.

crushed cells were suspended in 1 M citrate and again centrifuged  $g$  for 20 minutes. The supernatant 75-100 ml of 2 M NaCl in order to 1 to facilitate the dispersion. The

suspension was recentrifuged at  $25000 \times g$  for 20 minutes. The upper layer containing most of the DNA was pipetted off and slowly poured into 4 volumes of 95 per cent ethanol. The DNA precipitated as a white gelatinous mass which became filamentous when stirred with a glass rod the filaments winding around the rod. The material was dried on filter paper. Another two similar extractions of DNA with 0.1 M NaCl were made from the bacterial residue. The total quantity of precipitated DNA was then dissolved in about 100 ml of 2 M NaCl and left overnight.

The crude DNA preparation was further purified from protein by the addition of 0.1 per cent sodium desoxycholate (2 per cent solution in proportion 1:20). The mixture was allowed to stand for 2 hours at 37° C and 1 hour at 4° C and was then centrifuged at  $3900 \times g$  for 20 minutes. The supernatant liquid was poured into 4 volumes of sterile 95 per cent ethanol. As previously the precipitated DNA was removed and dissolved in a small volume of sterile 2 M NaCl.

In some cases this relatively crude preparation of DNA was purified from RNA by digestion with RNase (Worthington  $3 \times$  cryst. 50  $\mu$ g per ml solution of DNA) at 37° C for 1 hour and from protein by the addition of 0.1 per cent sodium desoxycholate at 37° C for 1 to 2 hours and at 4° C for another 1 to 2 hours. The mixture was centrifuged and the supernatant precipitated with ethanol and dissolved in sterile 2 M NaCl as before.

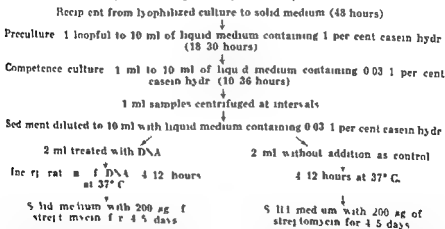
The concentration of the DNA solutions was determined by means of measuring their extinction at 260 m $\mu$  and 280 m $\mu$  in a Beckman DU spectrophotometer. The stock solutions had concentrations of about 1 mg DNA per ml. In connection with the experiments the preparations were diluted to suitable concentrations.

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Initially the transformation experiments were performed with the liquid medium that was later used only for DNA preparation. Under these experimental conditions resistant mutants were obtained only rarely.

An increase in the number of transformed bacteria was in some cases

observed if the recipient cells were treated with 0.5–1 IE penicillin 4 hours before the addition of the DNA.

In the transformation system of *B. subtilis* Spizizen (4) found that  $\text{Cu}^{++}$ , even in very small concentrations strongly diminishes the frequency of transformation and that the concentration of casein hydrolysate is critical. The original *B. pertussis* medium contained  $\text{CuSO}_4$  (0.5 mg per l) since  $\text{Cu}^{++}$  was supposed to be important for good growth. However  $\text{CuSO}_4$  could be omitted from the original medium without any decrease in growth. When it was found that the transformations obtained after this elimination were more frequent, no further experiments with penicillin-treated cells were carried out.

Different amounts of casein hydrolysate in concentrations from 0.03 per cent to 1 per cent in the competence culture and in the DNA-treated sample gave different amounts of transformed bacteria. The experiments showed that with 1 per cent casein hydrolysate, a concentration that provided good growth, no resistant cells occurred. However with 0.5 per cent or less casein hydrolysate transformed cells occurred. Even a culture with a concentration as low as 0.03 per cent casein hydrolysate permitted transformation, in spite of a very slow and limited growth. Table 1 illustrates experiments with different amounts of casein hydrolysate.

TABLE 1

*Effect of Different Amounts of Casein Hydrolysate and of Varying DNA Incorporation Time. 4 Experiments with Recipient 44122/7 Treated with about 1  $\mu\text{g}$  per ml of Purified DNA*

| Per cent casein hydrolysate | Resistant mutants per 0.5 ml of inoculum          |    |      |       |                   |   |   |    |
|-----------------------------|---|----|------|-------|-------------------|---|---|----|
|                             | Time between addition of DNA and plating in hours |    |      |       | Controls in hours |   |   |    |
|                             | 4   | 8  | 9    | 11    | 4                 | 6 | 9 | 11 |
| 1                           | 0   | 0  | 0    | 0     | 0                 | 0 | 0 | 0  |
| 0.5                         | 0   | 0  | >400 | >400* | 0                 | 0 | 0 | 0  |
| 0.25                        | 5   | 51 | >200 | >200  | 0                 | 0 | 0 | 0  |
| 0.12                        | 0   | 39 | 70   | 200   | 0                 | 0 | 0 | 0  |
| 0.06                        | 0   | 2  | 20   | 100   | 0                 | 0 | 0 | 0  |
| 0.03                        | 0   | 10 | 2    | 5     | 0                 | 0 | 0 | 0  |

\*The very rough estimate depends on the difficulty to spread the cells evenly as *B. pertussis* tends to grow as clusters in liquid media.

The pertussis bacteria were found to be competent for transformation during a rather long period, at least 18 to 20 hours. In rare instances competent bacteria were found in cultures about 10 hours old. From cultures of 20 to 27 hours age, however, the largest number and most constantly competent bacteria were found.

Only in rare instances and in a small number, resistant mutants were obtained if the samples were seeded on the solid medium containing streptomycin within 4 to 5 hours after the addition of DNA. In experi-

ments under similar conditions, resistant mutants were obtained if the interval was extended to 6 to 9 hours (Table 1).

Some of the resistant mutants which had grown on solid medium containing 200  $\mu$ g of streptomycin per ml were tested for their degree of resistance. They were all resistant to more than 5000  $\mu$ g of streptomycin per ml on solid medium. The resistance to streptomycin evidently was linked to another genetic marker, giving independence of starch since it was found that all transformed cells tested (about 20) were independent of starch in the growth medium.

## DISCUSSION

Transformation experiments performed with bacteria such as pneumococci (9), *H. influenzae* (3, 10), and *B. subtilis* (13) show that in these systems competence develops rather quickly toward the end of the logarithmic growth phase and disappears soon after the stationary growth phase is reached. The meningococci (6) constitute an exception as they are competent for a long time during their logarithmic growth phase.

The division time for *B. pertussis* exceeds by far that of other bacteria used in transformation experiments (it is about 3 hours in the medium used in these experiments). Therefore one can expect a longer duration and a less marked peak of competence than for other systems. The results of the experiments show a competence time extending over at least 18 hours.

In transformation experiments performed with pneumococci (8) and *H. influenzae* (2) the phenotypic delay is about 2 hours. If streptomycin resistance is used as marker the cells are insusceptible to streptomycin after this time. As the growth rate of *B. pertussis* is considerably slower than that of these bacteria, one can expect a longer interval before resistance is definitely established. This fact can explain that after 2 hours one can not demonstrate any resistant mutants at all, while after 4 hours only a few mutants are found. An interval of 8 to 12 hours seems to be necessary for a high yield of resistant cells.

The frequency of transformations has been low in most experiments but is nevertheless significant when seen against the fact that the spontaneous mutation rate of *B. pertussis* toward streptomycin-resistance is very low. According to Alexander *et al.* (1) it is in the magnitude of one per  $10^{10}$  to  $10^{11}$  bacteria per ml.

## SUMMARY

A streptomycin sensitive strain of *B. pertussis* has been transformed to streptomycin-resistance with DNA from a streptomycin-resistant mutant. The streptomycin-resistance seems to be linked to independence of starch in the growth medium.



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## RESISTANCE TO METHICILLIN, ISOXAZOLYL PENICILLINS, AND CEPHALOTHIN IN *STAPHYLOCOCCUS AUREUS*

By

KALD RIEWERTS FRIKSEN and INGRID FRICHSEN

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During the last few years a number of penicillinase resistant penicillins has become available for clinical use (7-23). Methicillin introduced in 1960 and the more recently introduced isoxazolyl compounds, oxacillin and nafcillin, have been used clinically in Denmark since 1960.

(Ampicillin\*) and nafcillin have been available for clinical trials in the United States (2, 22, 26, 27). Recently a new series of semisynthetic compounds closely related to penicillin, the cephalosporins, has received much interest (1, 7, 23). One of these preparations, cephalothin, has been subjected to clinical investigation in U.S.A. (27).

For all these compounds the rationale for their use in infections caused by penicillin resistant staphylococci is their resistance against the action of staphylococcal penicillinase. Especially with methicillin clinical experience is very large and this penicillin is generally considered the drug of choice for treatment of severe infections with penicillinase-producing staphylococci.

However, it is known from laboratory experiments that penicillinase-producing strains of *Staphylococcus aureus* can by subculturing in the presence of methicillin acquire an increased resistance to methicillin without loss of properties generally considered to be associated with virulence (8, 11, 30). Such strains show an increased resistance also against other penicillinase-resistant penicillins and the cephalosporins (6, 7, 30).

Naturally occurring strains of *Staphylococcus aureus* resistant to methicillin and to the other penicillinase resistant penicillins, including the cephalosporins, have been described by a number of authors (5, 7,

\* Semisynthetic penicillins were kindly supplied by H. Lundbeck & Co. A/S, Copenhagen.



Fig 1

Fig 1 Methicillin sensitive *Staphylococcus aureus*

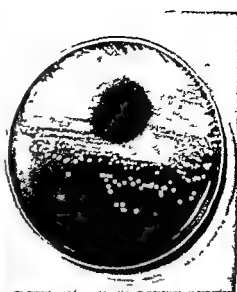


Fig 2

Fig 2 Methicillin resistant *Staphylococcus aureus*

8, 9, 10, 12, 17, 20, 21, 28, 29) since the first report by Jevons in 1961 (19) of methicillin-resistant strains isolated from clinical sources

In a previous paper (15), the isolation of methicillin-resistant strains of *Staphylococcus aureus* from two patients in a Danish hospital was reported. In this paper the isolation of similar strains from eight additional patients is reported together with some experimental findings concerning the nature of methicillin resistance.

## MATERIAL AND METHODS

The methicillin resistant strains of *Staphylococcus aureus* reported in this paper have been found either among pure cultures forwarded from dr Kirsten Rosendal Statens Seruminstitut or they have been isolated from material sent to the Institute of General Pathology for bacteriological diagnosis.

Testing for sensitivity to methicillin has been performed on all strains received or isolated in the laboratory since October 1960. Until the end of the year 1963 more than 14 000 strains isolated from clinical sources have been tested; strains isolated from healthy nasal carriers among hospital staffs not being included in this number.

Tests for methicillin sensitivity were made by the paper disc method described by Jensen & Kjær (18). The amount of methicillin used for each disc has been 50 µg. The disc (20 mm in diameter) is removed after 30 minutes and the plate is inoculated with a platinum loop in such a way that one third of the area covered by the disc is more heavily seeded than the rest.

By this method methicillin sensitive staphylococci after overnight incubation at 37° C show inhibition zones at least 40 mm in diameter (Fig 1). With a methicillin resistant strain the inhibition zone is usually not more than 25 mm in diameter (Fig 2). This method has been used in all cases in which a rapid sensitivity determination was required, i.e. before pure cultures of the staphylococci present in the specimen could be made.

As a routine however the test has been performed on pure cultures isolated from single colonies. From an overnight broth culture inoculation has been made in a streak at a right angle against the impression made by the edge of the removed paper disc.

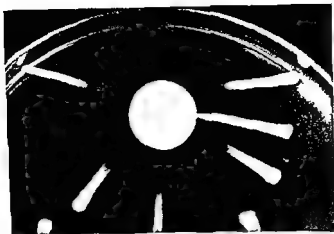


Fig 3

Streak plate with one methicillin resistant and five methicillin sensitive strains of *Staphylococcus aureus*. The original paper disc was removed before inoculation. A new disc has been applied because the impression made by the original disc is not easily seen in a photo.

With this method the inhibition zones for sensitive strains are 10 mm or more for resistant ones less than 5 mm (Fig 3). All strains found resistant by one of

part in an incubator at 37°C and read at the end of 24 and 48 hours for visible growth.

For each experiment the numbers of living staphylococcal cells in the cultures used as inoculum have been estimated by plating suitable dilutions and counting the numbers of colonies appearing after overnight incubation. The numbers have for all strains used been  $100-150 \times 10^6$  per ml.

In experiments with solid media nutrient agar was used. The plates were 8.5 cm in diameter. The inoculum in a volume of 0.1 ml was spread over the surface of the medium with a sterile Pasteur pipette. Colonies were counted after 24 and 48 hours incubation at 37°C.

Inactivation of the various compounds was tested in 0.1 ml broth cultures inoculated with undiluted broth culture as described above. After incubation for varying periods at 37°C the penicillinase effect was interrupted by filtration through a No. 1 filter. The entire culture was used for this purpose. Thus in each experiment a number of single cultures was used. The cultures were however prepared in a way that

in one experiment being not identical just because of the slight differences in the amount of the experimental material.

Interpretation of drug action in the Seitz filtered cultures was made by the agar dilution method described by Osterholm (31). As test organism *Staphylococcus aureus* ATCC 29101 was used.

Penicillinase production has been demonstrated by a modified C<sub>12</sub> technique



Fig 1

Fig 1 Methicillin sensitive *Staphylococcus aureus*

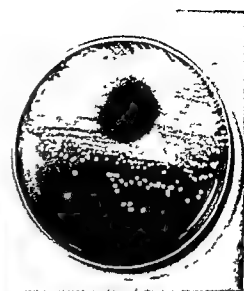


Fig 2

Fig 2 Methicillin resistant *Staphylococcus aureus*

8, 9, 10, 12, 17, 20, 21, 28, 29) since the first report by Jevons in 1961 (19) of methicillin-resistant strains isolated from clinical sources

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By this method methicillin sensitive staphylococci after over night incubation at 37° C show inhibition zones at least 40 mm in diameter (Fig 1). With a methicillin resistant strain the inhibition zone is usually not more than 25 mm in diameter (Fig 2). This method has been used in all cases in which a rapid sensitivity determination was required, i.e. before pure cultures of the staphylococci present in the specimen could be made

As a routine, however the test has been performed on pure cultures isolated from single colonies. From an overnight broth culture inoculation has been made in a streak at a right angle against the impression made by the edge of the removed paper disc

TABLE I  
Sources of Methicillin Resistant Strains of *Staphylococcus aureus*

| Case no. | Hospital | Date of first isolation of the methicillin resistant strain | Material from which the strain was isolated | Sensitivity pattern | Isolate type       | Antibiotic treatment prior to first isolation of the methicillin resistant strain |
|----------|----------|---|---|---------------------|--------------------|---|
| 1        | A        | Aug 1962  | sputum urine pus from abscess               | PST                 | 6/75/77            | unknown   |
| 2        | A        | Aug 1962  | tracheal aspirate                           | PST                 | 6/75/77            | no treatment  |
| 3        | B        | June 1963   | pus from fistula cuff and from tracheostomy | PSTII               | 47/54/77           | penicillin chlorotetracycline   |
| 4        | C        | July 1963   | pus from post operative wound infection     | PSTI                | N.T.               | penicillin streptomycin   |
| 5        | D        | Nov 1963  | pus from lung abscess                       | PSTII               | 7/47/54/77         | penicillin oxytetracycline streptomycin   |
| 6        | I        | Dec 1963  | pus from post operative wound infection     | PSTII               | 47/54/77           | erythromycin chlorotetracycline   |
| 7        | D        | Dec 1963  | sputum                                      | PSTII               | 47/54/77 +         | penicillin  |
| 8        | D        | Dec 1963  | sputum                                      | PSTI                | 7/47/54/77         | penicillin  |
| 9        | I        | Dec 1963  | pus from pleural empyema                    | PSTP                | 7/47/53/54/77/83 A | penicillin streptomycin rotitetracycline  |
| 10       | I        | Dec 1963  | pus from post operative wound infection     | PSTII               | 7/47/54/77 +       | penicillin streptomycin   |

PSTI Resistant to penicillin streptomycin, tetracyclines and erythromycin

PST Resistant to penicillin streptomycin and tetracyclines

using agar containing 0.04 units/ml of penicillin G and as test organism a highly penicillin-sensitive strain of *B. subtilis* (14/16).

*Penicillinase-negative mutants* were isolated from broth cultures kept for 2-3 weeks at room temperature. After subculturing on agar plates 50 separate colonies were tested for penicillinase production. From colonies which appeared negative at *Gates* plates broth cultures were made and tested once more for lack of penicillinase activity by overnight incubation in broth with 1 unit of penicillin G per ml.

*Phage typing* was performed in Statens Seruminstitut, Copenhagen.

The methicillin-resistant *Staphylococcus aureus* 55974 used in most experiments was isolated from case 1 (Table 1).

The methicillin-sensitive strains of *Staphylococcus aureus* used in various experiments were

326 a non penicillinase-producing strain fully sensitive to penicillin G, phage-type 80/81,

2719 a potent penicillinase-producer, phage type 80/81

1674 a potent penicillinase producer with a high tendency to split off penicillinase-negative variants phage type 80/81

## RESULTS

Methicillin-resistant strains of *Staphylococcus aureus* have been isolated from ten patients admitted to six different hospitals in Copenhagen. From most cases the resistant strain has been isolated on more than one occasion. Table 1 shows, for each patient, the sites from which the methicillin-resistant strain was isolated together with the date for its first isolation. The hospital to which the patient was admitted when the presence of methicillin-resistant staphylococci was first recognized is indicated by a capital.

*Phage-types and sensitivity-patterns* are shown in Table 1. All strains were resistant to penicillin, streptomycin, and tetracyclines and, with the exception of the strains isolated from cases 1 and 2, also to erythromycin. They were sensitive to chloramphenicol, neomycin, kanamycin, and fusidic acid.

One strain (case 4) was non-typable, all other strains belonged to phage group III. The strains from cases 1 and 2 appeared identical. The strains from the remaining seven cases showed identical or closely related phage-typing patterns, but were distinctly different from the strains isolated from cases 1 and 2.

### *Susceptibility to Methicillin, Oxacillin, Cloxacillin, Cephalothin, and Penicillin G*

All strains found methicillin-resistant by the disc method have been tested in fluid medium against graded concentrations of methicillin, using an inoculum of 0.5 ml of an undiluted over-night broth culture. After 24 hours incubation at 37° C they all grow in 10 µg/ml. After 48 hours incubation all strains grow in 500, some even in 1000 µg/ml. However, no significant difference in sensitivity of various strains could be

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We are grateful to Kirsten Rosendal MD for typing a large number of strains

demonstrated in one experiment a strain may grow in 1000 in another one only in 200  $\mu\text{g/ml}$

*Staphylococcus aureus* S 5974 isolated from case 1 has been subjected to further studies. Its susceptibility against all four penicillins and cephalothin has been tested in fluid medium using various inocula and has been compared with that of the methicillin sensitive penicillinase producing *Staphylococcus aureus* 2719

It is evident from the results of the experiments with the three penicillinase resistant penicillins (Table 2) that the susceptibility of staphylococcus S 5974 against all these compounds is significantly lower than that of staphylococcus 2719

The resistant strain shows however a rather significant inoculum size effect. The susceptibility of the smallest inocula employed is in fact only insignificantly lower than that of the sensitive strain. This phenomenon is most marked with methicillin and cloxacillin

In the case of methicillin the inoculum effect observed contrasts sharply to the findings with the sensitive strain which does not show any real inoculum effect at all. However with oxacillin and cloxacillin also the sensitive strain exhibits a certain inoculum effect due to the fact that these penicillins are less resistant against penicillinase than is methicillin (6)

Also against cephalothin the susceptibility of the resistant strain is significantly lower than that of the sensitive strain (Table 3). The observed difference is however not significant. Also the sensitivity of the sensitive strain is in the same degree as that of the resistant strain

The susceptibility to penicillin G (Table 4) is likewise lower for the methicillin resistant strain than for the sensitive strain but because of the high penicillinase activity of the methicillin sensitive strain the difference between the two strains is not significant when comparing cultures seeded with very large inocula. With the smallest inoculum used however the difference between the two strains is very significant the sensitivity of the resistant strain being 8-16 times lower than that of the sensitive one

With the same technique is used in the experiments described above two other strains 1739 and 1743 isolated from cases 3 and 4 respectively have been tested against methicillin, oxacillin and penicillin G. The results of these experiments were virtually identical to those obtained with staphylococcus S 5974

#### *Inactivation of Methicillin and Oxacillin during Growth of the Methicillin Resistant Staphylococcus*

In an attempt to elucidate the mechanism of the rather marked inoculum size-effect of the methicillin resistant strain subcultures were made from cultures grown in broth with 100  $\mu\text{g/ml}$  of methicillin. These





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Also against cephalothin the susceptibility of the resistant strain is significantly lower than that of the sensitive strain (Table 3). The same inoculum effect as with the penicillins is observed. Also the sensitive strain shows an inoculum effect of nearly the same degree as that seen with oxacillin and cloxacillin.

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With the same technique as used in the experiments described above two other strains 1799 and 1/43 isolated from cases 3 and 4 respectively have been tested against methicillin, oxacillin and penicillin G. The results of these experiments were virtually identical to those obtained with staphylococcus S 5974.

#### *Inactivation of Methicillin and Oxacillin during Growth of the Methicillin Resistant Staphylococcus*

In an attempt to elucidate the mechanism of the rather marked inoculum size effect of the methicillin resistant strain subcultures were made from cultures grown in broth with 100  $\mu\text{g/ml}$  of methicillin. These

TABLE 3  
*Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus to Cephalothin*

| Cephalothin (one in $\mu\text{g/ml}$ ) | Methicillin resistant <i>Staph. aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs. culture |                 |                 |                 |                 |                 | Methicillin sensitive <i>Staph. aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs. culture |                 |                 |                 |                 |                 |
|--|---|-----------------|-----------------|-----------------|-----------------|-----------------|---|-----------------|-----------------|-----------------|-----------------|-----------------|
|  | 10 <sup>1</sup>   | 10 <sup>2</sup> | 10 <sup>3</sup> | 10 <sup>5</sup> | 10 <sup>7</sup> | 10 <sup>9</sup> | 10 <sup>1</sup>   | 10 <sup>2</sup> | 10 <sup>3</sup> | 10 <sup>5</sup> | 10 <sup>7</sup> | 10 <sup>8</sup> |
| 100                                    | —   | —               | —               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 50                                     | —   | —               | —               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 25                                     | +   | +               | +               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 10                                     | +   | +               | +               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 5                                      | +   | +               | +               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 2.5                                    | +   | +               | +               | —               | —               | —               | —   | —               | —               | —               | —               | —               |
| 1.25                                   | +   | +               | +               | +               | —               | —               | —   | —               | —               | —               | —               | —               |
| 0.625                                  | +   | +               | +               | +               | +               | —               | —   | —               | —               | —               | —               | —               |
| 0.312                                  | +   | +               | +               | +               | +               | +               | —   | —               | —               | —               | —               | —               |
| 0.156                                  | +   | +               | +               | +               | +               | +               | +   | +               | +               | +               | +               | +               |
| 0.078                                  | +   | +               | +               | +               | +               | +               | +   | +               | +               | +               | +               | +               |

+ + growth after 24 hours incubation      - + growth after 48 hours incubation      — — no growth

TABLE 4  
Susceptibility of a Methicillin Resistant and a Methicillin Sensitive *Penicillinae* Producing *Staphylococcus aureus* to Penicillin G

| Penicillin G (one in µg/ml)         | Methicillin resistant <i>Staph aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs culture |                 |                 |                 |                 | Methicillin sensitive <i>Staph aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs culture |                 |                 |                 |                 |
|-------------------------------------|---|-----------------|-----------------|-----------------|-----------------|---|-----------------|-----------------|-----------------|-----------------|
|                                     | 10 <sup>1</sup>   | 10 <sup>2</sup> | 10 <sup>5</sup> | 10 <sup>7</sup> | 10 <sup>8</sup> | 10 <sup>1</sup>   | 10 <sup>2</sup> | 10 <sup>5</sup> | 10 <sup>7</sup> | 10 <sup>8</sup> |
| 10000                               | +   | +               | —               | —               | —               | +   | +               | —               | —               | —               |
| 1000                                | +   | +               | —               | —               | —               | +   | +               | —               | —               | —               |
| 100                                 | +   | +               | —               | —               | —               | +   | +               | —               | —               | —               |
| 10                                  | +   | +               | —               | —               | —               | +   | +               | —               | —               | —               |
| 5                                   | +   | +               | +               | +               | +               | +   | +               | —               | —               | —               |
| 2.5                                 | +   | +               | +               | +               | +               | +   | +               | —               | —               | —               |
| 1.25                                | +   | +               | +               | +               | +               | +   | +               | —               | —               | —               |
| 0.625                               | +   | +               | +               | +               | +               | +   | +               | +               | +               | +               |
| 0.312                               | +   | +               | +               | +               | +               | +   | +               | +               | +               | +               |
| ++ growth after 24 hours incubation |   |                 |                 |                 | — no growth     |   |                 |                 |                 |                 |



TABLE 4  
*Susceptibility of a Methicillin Resistant and a Methicillin Sensitive Penicillinase Producing Staphylococcus aureus to Penicillin G*

| Penicillin G Conc in $\mu\text{g/ml}$ | Methicillin resistant <i>S. a. aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs culture |        |        |        |        |        | Methicillin sensitive <i>S. a. aureus</i><br>Initial number of staphylococci per ml<br>as concentration of 24 hrs culture |        |        |        |        |        |
|---------------------------------------|---|--------|--------|--------|--------|--------|---|--------|--------|--------|--------|--------|
|                                       | $10^1$  | $10^2$ | $10^3$ | $10^5$ | $10^7$ | $10^8$ | $10^1$  | $10^2$ | $10^3$ | $10^5$ | $10^7$ | $10^8$ |
| 10000                                 | +   | +      | —      | —      | —      | —      | +   | +      | —      | —      | —      | —      |
| 1000                                  | +   | +      | —      | —      | —      | —      | +   | +      | —      | —      | —      | —      |
| 100                                   | +   | +      | +      | —      | —      | —      | +   | +      | +      | —      | —      | —      |
| 10                                    | +   | +      | +      | +      | —      | —      | +   | +      | +      | —      | —      | —      |
| 5                                     | +   | +      | +      | +      | +      | —      | +   | +      | +      | —      | —      | —      |
| 2.5                                   | +   | +      | +      | +      | +      | +      | +   | +      | +      | —      | —      | —      |
| 1.25                                  | +   | +      | +      | +      | +      | +      | +   | +      | +      | +      | —      | —      |
| 0.625                                 | +   | +      | +      | +      | +      | +      | +   | +      | +      | +      | +      | —      |
| 0.312                                 | +   | +      | +      | +      | +      | +      | +   | +      | +      | +      | +      | +      |

|   |                                  |    |   |                                  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
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+ + growth after 24 hours incubation    -- + growth after 48 hours incubation    -- -- no growth

subcultures were influenced by methicillin in exactly the same way as the original culture. It thus appeared probable that the inoculum effect might be due to inactivation of methicillin (15).

That methicillin is actually inactivated during growth of the methicillin-resistant strain has been shown in experiments in which cultures of staphylococcus S 5974 in broth with respectively 10 and 100  $\mu\text{g/ml}$  of methicillin were tested for methicillin-activity after growth for various periods of time.

In tubes with an initial concentration of 10  $\mu\text{g/ml}$  inoculated with 0.5 ml of an undiluted culture the concentration of methicillin was after 10 hours incubation at 37° C 5, and after 18 hours 2.5  $\mu\text{g/ml}$ .

With an initial concentration of 100  $\mu\text{g/ml}$  the activity after 24 hours was 10-25 and after 48 hours less than 2  $\mu\text{g/ml}$ .

Also oxacillin is inactivated during growth of the resistant strain. In tubes with an initial concentration of 10  $\mu\text{g/ml}$  the concentration after 6 hours incubation was less than 0.3. After 6 hours incubation of cultures with an initial concentration of 100  $\mu\text{g/ml}$  the activity corresponded to 5-2.5  $\mu\text{g/ml}$  and after 12 hours incubation the concentration was only 0.3  $\mu\text{g/ml}$ .

In all experiments of this kind tubes with uninoculated broth containing the same concentrations of drug as used in the inactivation test have been incubated and have been tested for activity after filtration through a Seitz filter. No loss of activity has been observed.

The methicillin-inactivating properties of all other methicillin-resistant strains have been tested. All strains inactivate methicillin to virtually the same degree.

It thus appears as if the inoculum effect is, at least partly, a result of inactivation of methicillin.

### *Relationship between Resistance to Methicillin and Production of Penicillinase*

In order to examine further the relationship between methicillin resistance and the inactivation of methicillin the sensitivity of the resistant staphylococcus S 5974 has been compared to that of a penicillinase-negative strain derived from the original methicillin-resistant one. This strain showed the same phage-type and sensitivity-pattern as the original strain S 5974.

In broth the penicillinase-negative strain showed a significantly lower resistance to methicillin than did the original strain (Table 5). In tubes inoculated with 0.5 ml of an undiluted culture growth occurred after 24 hours in 10  $\mu\text{g/ml}$  and after 48 hours in 100  $\mu\text{g/ml}$ , while the original strain grew in 100 and 600  $\mu\text{g/ml}$  respectively. With smaller inocula growth was seen in 1.25  $\mu\text{g/ml}$ , the original strain growing in 2.5-5  $\mu\text{g/ml}$ .

Thus, also with this penicillinase negative strain a certain inoculum

TABLE 5

*Susceptibility of the Parent Strain and a Penicillinase Negative Mutant of a Methicillin Resistant Staphylococcus aureus to Methicillin*

| Methicillin conc. in $\mu\text{g}/\text{ml}$ | Parent strain  |        |        |        |        | Penicillinase negative mutant  |        |        |        |        |
|--|--|--------|--------|--------|--------|--|--------|--------|--------|--------|
|  | Initial number of staphylococci per ml as concentration of 24 hrs. culture |        |        |        |        | Initial number of staphylococci per ml as concentration of 24 hrs. culture |        |        |        |        |
|  | $10^1$   | $10^2$ | $10^5$ | $10^7$ | $10^8$ | $10^1$   | $10^2$ | $10^5$ | $10^7$ | $10^9$ |
| 800  | —  | —      | —      | —      | —      | —  | —      | —      | —      | —      |
| 600  | —  | —      | —      | —      | —      | —  | —      | —      | —      | —      |
| 400  | +  | —      | —      | —      | —      | —  | —      | —      | —      | —      |
| 200  | +  | —      | —      | —      | —      | —  | —      | —      | —      | —      |
| 100  | +  | +      | —      | —      | —      | +  | —      | —      | —      | —      |
| 50   | +  | +      | —      | —      | —      | +  | —      | —      | —      | —      |
| 25   | +  | +      | —      | —      | —      | +  | +      | —      | —      | —      |
| 10   | +  | +      | +      | —      | —      | +  | +      | —      | —      | —      |
| 5  | +  | +      | +      | +      | +      | +  | +      | —      | —      | —      |
| 2.5  | +  | +      | +      | +      | +      | +  | +      | +      | +      | +      |
| 1.25   | +  | +      | +      | +      | +      | +  | +      | +      | +      | +      |

++ growth after 24 hours incubation    — + growth after 48 hours incubation    — no growth



effect was observed, but less marked than for the parent strain and the inhibitory concentrations were, with all inocula, significantly lower than for the original strain

This experiment has clearly shown that the original methicillin-resistant staphylococcus is significantly more resistant to methicillin than is the penicillinase-negative strain derived from it

The association between loss of penicillinase activity and increased susceptibility to methicillin is, perhaps, even better demonstrated by comparing the growth of the two strains on methicillin-containing agar plates

With an inoculum of 0.1 ml undiluted broth culture of the original strain confluent growth is obtained in 24 hours at concentrations up to 10  $\mu\text{g/ml}$ . At plates with 100  $\mu\text{g/ml}$  about 200 very small colonies are seen after 24 hours. After 1 or 2 days further incubation most of them have grown out to large, pigmented colonies with a distinct tendency to occur in clumps (Fig. 4). The total number of colonies does usually not increase significantly above the number seen after 24 hours, except for a varying number of small colonies which may sometimes grow out around the larger colonies.

With the penicillinase-negative strain, however, confluent growth is not seen at concentrations higher than 1.25 or 2.5  $\mu\text{g/ml}$ . At 10  $\mu\text{g/ml}$  the number of colonies after 24 hours is about 100, all colonies being very small. During the next days more colonies appear and after 3 days incubation the number is 200 or more. At 100  $\mu\text{g/ml}$  no growth is seen after 24 hours, after 48 hours about 25 very small colonies have grown out and after 3 days incubation the number of colonies is about 100. The colonies do not tend to be clumped together, as was the case with the original strain. After 3 days incubation the colonies, which first appeared, have grown out to large, pigmented ones. The youngest colonies are small and unpigmented, and all possible intermediate forms between these and the large ones are seen (Fig. 5).

The growth of the penicillinase-negative strain on agar with 100  $\mu\text{g/ml}$  of methicillin is, thus, significantly slower than that of the original strain and the appearance of colonies is much more similar to that of penicillin-resistant colonies grown out on penicillin-containing agar plates seeded with a large inoculum of a penicillin-sensitive staphylococcus.

When using, for comparison, the highest concentrations permitting confluent growth, the penicillinase-negative strain shows a susceptibility to methicillin 4–8 times higher than that of the original strain.

Also with an inoculum of 0.1 ml of a  $10^{-6}$  dilution of a broth culture (100–150 bacteria) the difference between the two strains is very significant.

In Table II the minimal inhibitory concentrations as measured by this method after incubation for 24 and 48 hours, respectively, are given. For comparison the corresponding values for two methicillin-sensitive strains are also given. For each strain the numbers of colonies

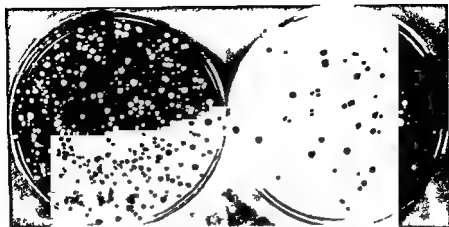


Fig 4

Fig 5

Fig 4 Plate with nutrient agar containing 100  $\mu$ g/ml of methicillin inoculated with 0.1 ml of broth culture of the methicillin resistant *Staphylococcus aureus* S 5974. Incubated for 72 hours at 37° C.

Fig 5 Plate with nutrient agar containing 100  $\mu$ g/ml of methicillin inoculated with 0.1 ml of broth culture of a penicillinase negative strain derived from *Staphylococcus aureus* S 5974. Incubated for 72 hours at 37° C.

on all concentrations permitting growth are virtually identical all cells in the inoculum growing out to colonies. The colonies appearing after 24 hours on the highest concentration are often small and unpigmented and growth may, as indicated in the table, be visible first after 48 hours incubation. After 72 hours, however, the colonies on all concentrations for all strains appear as typical pigmented staphylococcal colonies.

Thus it appears also from this experiment that the penicillinase negative strain is more sensitive to methicillin than the original methicillin resistant strain.

The experimental results given in Table II have further shown that the growth of the penicillinase-negative strain is inhibited by a concentration of methicillin only double as high as the concentration which inhibits the growth of a methicillin sensitive staphylococcus.

Several other penicillinase negative strains (all of identical phage type and sensitivity pattern) derived from *Staphylococcus aureus* S 5974 have been tested with the same technique. The results have always been virtually the same as those given in Table 6.

#### *Susceptibility of the Methicillin Resistant Staphylococcus to Penicillin G*

From experiments in which the susceptibility to penicillin G was estimated in fluid medium it appeared probable that the methicillin resistant staphylococcus S 5974 had an inherent resistance to penicillin (Table 4). However no certain conclusions can be drawn from

experiments of that kind because inactivation by penicillinase may play a rôle even when the inoculum is very small. With the isolation of penicillinase-negative strains, however, a possibility was offered for proving the existence of the "inherent" penicillin-resistance suggested.

Penicillinase-negative strains derived from a penicillinase-producing *Staphylococcus aureus* have usually been found fully sensitive to penicillin (4). Therefore, the strains have been compared with a penicillin-sensitive staphylococcus and with a penicillinase-negative strain derived from a methicillin-sensitive penicillinase-producing staphylococcus. Unfortunately, no penicillinase-negative strains could be isolated from staphylococcus 2719 used in other experiments. Staphylococcus 1634 used in this experiment is, as staphylococcus 2719, a potent penicillinase-producer and it shows the same degree of resistance to penicillin G.

TABLE 6

*Susceptibility to Methicillin of a Methicillin Resistant Staphylococcus aureus, a Penicillinase-Negative Strain derived from it and of two Methicillin Sensitive Strains of Staph. aureus. Estimated on Solid Medium Inoculated with 0.1 ml of a  $10^{-1}$  Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

|  | Minimal inhibitory concentrations of methicillin in $\mu\text{g/ml}$ |                   |
|--|--|-------------------|
|  | 24 hrs incubation  | 48 hrs incubation |
| Methicillin resistant <i>Staph. aureus</i> S 5974                              | 10   | 10                |
| Penicillinase negative strain (No. 1) derived from <i>Staph. aureus</i> S 5974 | 2.5  | 5                 |
| Methicillin sensitive penicillinase producing <i>Staph. aureus</i> 2719        | 1.25   | 2.5               |
| Methicillin sensitive non penicillinase-producing <i>Staph. aureus</i> 326     | 1.25   | 1.25              |

(Colonies counted after 24 and 48 hours incubation at  $37^{\circ}\text{C}$ )

TABLE 7

*Susceptibility to Penicillin G of a Penicillin-Sensitive Staphylococcus aureus and of Penicillinase Negative Strains Derived from a Methicillin Resistant and from a Methicillin Sensitive Staphylococcus aureus. Estimated on Solid Medium Inoculated with 0.1 ml of a  $10^{-1}$  Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

|  |                                       | Minimal inhibitory concentrations of penicillin G in $\mu\text{g/ml}$ |                   |
|--|---------------------------------------|---|-------------------|
|  |                                       | 24 hrs incubation   | 48 hrs incubation |
| Penicillinase negative strains   | No. 1                                 | 0.08  | 0.156             |
|  | No. 2                                 | 0.08  | 0.156             |
|  | resistant <i>Staph. aureus</i> S 5974 | 0.156   | 0.312             |
| Penicillinase negative strain derived from methicillin sensitive <i>Staph. aureus</i> 1634 |                                       | 0.02  | 0.02              |
| Penicillin sensitive <i>Staph. aureus</i> 326  |                                       | 0.02  | 0.02              |

(Colonies counted after 24 and 48 hours incubation at  $37^{\circ}\text{C}$ )

The results of an experiment, in which the susceptibility to penicillin G of these strains have been determined on solid media, are given in Table 7. As in the experiment with methicillin (Table 6) all cells in the inoculum give rise to colonies, if growth occurs at all.

This experiment has shown that the penicillinase-negative strain derived from the methicillin sensitive, penicillinase-producing staphylococcus is, as might be expected, fully sensitive to penicillin G. The three penicillinase negative strains derived from staphylococcus S 5974, however, are 4-8 times or, if the susceptibility is estimated from the growth after 48 hours incubation, 8-16 times more resistant to penicillin G than the sensitive staphylococcus.

### DISCUSSION

The methicillin resistant strains of *Staphylococcus aureus* described in this paper appear to be nearly related to the naturally occurring methicillin-resistant strains described by other authors. Except for the strain isolated from case 4, which was non-typable, all have belonged to phage group III and the phage-typing patterns were very similar to those of the majority of other reported strains. Also the sensitivity-patterns have been closely related to those reported by other authors, all strains hitherto reported being resistant to streptomycin and tetracyclines, many also to erythromycin (6, 7, 10, 20).

As to the degree of resistance to methicillin the Danish strains are obviously, closely related to the "Carshalton-strain" described by Stewart & Holt (29). *Staphylococcus* S 5974, which has been subjected to closer studies, appears also in respect to inoculum size effect, to resistance against the isoxazolyl penicillins and to inactivation of these penicillins to behave in the same manner as the "Carshalton-strain". Only regarding the inactivation of methicillin are the results obtained with the Danish strains not in agreement with the results of these authors who did not find any inactivation of methicillin by the strains how sent.

In addition to the methicillin resistant staphylococci have subsequently been observed by Ayliffe & Barber (3) working with, among other strains S 5974 used in the experiments reported above. The findings by Stewart & Holt concerning inactivation of cloxacillin have been confirmed by Knox & Smith (25).

Stewart & Holt (29) emphasized that their "Carshalton-strains" differed in several ways from the first, naturally resistant strains reported by Jevons (19), the main difference being that the strains of Jevons contained a mixed population of cells the majority of which were sensitive to methicillin.

However, Jevons *et al.* (20) do not emphasize this difference, despite

experiments of that kind because inactivation by penicillinase may play a rôle even when the inoculum is very small. With the isolation of penicillinase negative strains, however, a possibility was offered for proving the existence of the "inherent" penicillin-resistance suggested.

Penicillinase negative strains derived from a penicillinase producing *Staphylococcus aureus* have usually been found fully sensitive to penicillin (4). Therefore, the strains have been compared with a penicillin-sensitive staphylococcus and with a penicillinase-negative strain derived from a methicillin sensitive penicillinase-producing staphylococcus. Unfortunately, no penicillinase-negative strains could be isolated from staphylococcus 2719 used in other experiments. *Staphylococcus* 1634 used in this experiment is, as staphylococcus 2719, a potent penicillinase-producer and it shows the same degree of resistance to penicillin G.

TABLE 6

*Susceptibility to Methicillin of a Methicillin Resistant Staphylococcus aureus a Penicillinase Negative Strain derived from it and of two Methicillin Sensitive Strains of Staph aureus Estimated on Solid Medium Inoculated with 0.1 ml of a  $10^8$  Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

|  | Minimal inhibitory concentrations of methicillin in $\mu\text{g/ml}$ |                   |
|--|--|-------------------|
|  | 24 hrs incubation  | 48 hrs incubation |
| Methicillin resistant <i>Staph aureus</i> S 5974                             | 10   | 10                |
| Penicillinase negative strain (No 1) derived from <i>Staph aureus</i> S 5974 | 2.5  | 5                 |
| Methicillin sensitive penicillinase-producing <i>Staph aureus</i> 2719       | 1.25   | 2.5               |
| Methicillin sensitive non penicillinase-producing <i>Staph aureus</i> 326    | 1.25   | 1.25              |

(Colonies counted after 24 and 48 hours incubation at 37° C)

TABLE 7

*Susceptibility to Penicillin G of a Penicillin Sensitive Staphylococcus aureus and of Penicillinase Negative Strains Derived from a Methicillin Resistant and from a Methicillin Sensitive Staphylococcus aureus Estimated on Solid Medium Inoculated with 0.1 ml of a  $10^8$  Dilution of a 18 Hours Broth Culture (100-150 Bacteria)*

|  |      | Minimal inhibitory concentrations of penicillin G in $\mu\text{g/ml}$ |                   |
|--|------|---|-------------------|
|  |      | 24 hrs incubation   | 48 hrs incubation |
| Penicillinase negative strains                 | No 1 | 0.08  | 0.156             |
| derived from methicillin                       | No 2 | 0.08  | 0.156             |
| resistant <i>Staph aureus</i> S 5974           | No 3 | 0.156   | 0.312             |
| Penicillinase negative strain derived from     |      |   |                   |
| methicillin sensitive <i>Staph aureus</i> 1634 |      | 0.02  | 0.02              |
| Penicillin sensitive <i>Staph aureus</i> 326   |      | 0.02  | 0.02              |

(Colonies counted after 24 and 48 hours incubation at 37° C)

to be readjusted in as much as the inactivation observed must necessarily cause some inoculum effect

Experiments reported above, with penicillinase negative strains derived from a methicillin resistant staphylococcus have shown such strains to be significantly more sensitive to methicillin than the original penicillinase producing strain

Penicillinase negative strains derived from penicillinase producing staphylococci usually possess all characteristics of the parent strain except penicillinase production. There is no reason for questioning that this was true also for the strains used in these experiments. They were all of the same phage type as the original strain and growth rate and appearance of colonies were likewise unchanged. In our opinion it is therefore justified to conclude from these experiments that methicillin resistance of the type described in this paper, and the inoculum effect characteristic of this kind of resistance, is at least partly a result of methicillin inactivation.

Certainly also with the penicillinase negative strains some inoculum effect was observed. Rather than concluding that such strains represent mixed cultures this phenomenon may perhaps better be explained as the results of a very great tendency for resistant cells to arise during incubation on methicillin containing medium.

That the appearance of colonies on methicillin containing agar inoculated with a penicillinase negative strain actually is very much like that of colonies grown out on penicillin containing agar inoculated with a fully sensitive staphylococcus appears evident when Fig 5 in the present paper is compared with the photo published in *Eriksen's* paper on the mode of origin of penicillin resistant staphylococci (13). The somewhat different appearance of growth on methicillin plates inoculated with the original methicillin resistant strain, especially the more rapid development of colonies which tend to be clumped together may easily be explained as the result of a superimposed effect of penicillinase.

Comparative investigations of penicillinase negative strains and the parent strain are continuing. One problem to be investigated is that of the sensitivity to methicillin of staphylococci growing out on methicillin containing medium. Preliminary experiments have shown that it is possible to select from a penicillinase negative strain after one passage on agar with 100  $\mu$ g methicillin per ml coagulase positive staphylococci of unchanged phage typing pattern which grow without any significant inoculum effect in broth with 1000  $\mu$ g/ml of methicillin.

Unfortunately we have not yet succeeded in selecting penicillinase negative mutants from other strains of methicillin resistant staphylococci. Therefore our conclusions must be taken with some caution being subject to the reservations that they were drawn from experiments with one single strain of *Staphylococcus aureus*.

With the possible exception of case I in which no information about

the fact that many of the methicillin-resistant strains described in their paper were actually those described by *Stewart & Holt* and others were received from the hospital in which the strains reported by *Jevons* in 1961 were isolated. They only mention that most resistant cultures grew continuously up to the methicillin-disc used in their plates and that a few, which contained a relatively small proportion of resistant individuals, showed only discrete colonies near the disc.

As a given strain may, undoubtedly, from day to day, show minor variations in its resistance to methicillin it seems unwarranted to draw any conclusions from differences of this kind. It appears more relevant to emphasize the great similarities between most clinically occurring methicillin-resistant strains hitherto described.

In fact, the experiments of *Knox & Smith* (24) with one of the strains described by *Jevons* in 1961 indicate a close similarity between this strain and the strains described in the present paper. Using a very small inoculum (about 10 bacteria) these authors found that the minimum inhibitory concentration of methicillin was four times greater for the resistant strain than for a methicillin sensitive penicillinase-producing strain, and they further found evidence of an "inherent" resistance to penicillin G. It is likewise worth mentioning that the strain 16137/1000 which by *Knox & Smith* (25) now is found to inactivate cloxacillin and methicillin was found to cause no inactivation.

A characteristic of naturally occurring methicillin resistant staphylococci, mentioned by several authors, is the marked inoculum-size-effect (6). Also the strains described in this paper have shown a significant inoculum effect. This is evident from the experiments carried out with staphylococcus S 5974, experiments which have been repeated with two other strains with virtually the same results as those reported in Table 2 and 3.

The somewhat irregular shape of the inhibition zones shown by resistant strains (Fig 2) is a clear illustration of the inoculum effect, the diameter of the zone being smallest in the most heavily seeded part of the field. In the streak method (Fig 3) the inoculum used has been proportionately very large, and the resistant strains are best recognized by this method. In fact, if the inoculum is too small, methicillin-resistance may not be recognized at all. Ignorance of this is probably the cause of the deplorable fact that some methicillin resistant strains described in this paper have been reported by other laboratories as being fully sensitive to methicillin.

It has, so far, been generally agreed that the inoculum effect is not due to any inactivation of methicillin and that this effect is adequately accounted for by assuming that a culture of a methicillin-resistant staphylococcus contains a mixed population of cells of which only a minority is highly resistant (6, 24).

After the demonstration of a significant inactivation of methicillin during growth of a methicillin resistant staphylococcus this view has

in Denmark, of methicillin resistant strains of *Staphylococcus aureus* is far from being negligible. The information given in the table suggest that the occurrence of these strains is partly a result of cross-infection and further epidemiological analysis has shown that the strains isolated from six cases in 1963 were, presumably, of common origin.

Case 5, from which two other patients (cases 7 and 8) in hospital D were cross infected, had been transferred to this hospital from a department for general surgery and were infected already while staying there. Case 3 had obviously acquired the infection in this department too. The strains from cases 9 and 10 were found among a collection of strains, kindly sent to us from dr. Kirsten Rosendal. They were isolated from specimens received from the same surgical department (hospital F) in which cases 3 and 5 acquired their infections.

After the completion of the present paper an investigation has been commenced in this department. Four nurses were found to carry the methicillin-resistant staphylococcus in their noses and from six patients with postoperative wound infections the same strain was isolated from pus. It is noteworthy that penicillinase resistant penicillins have been used in this department only in exceptional cases. It appears quite evident that the spread of the resistant strain in this department had no relationship whatever to the use of methicillin or other new penicillins. Details about the results of these investigations will be published elsewhere.

## SUMMARY

Methicillin-resistant strains of *Staphylococcus aureus* have been isolated from ten patients admitted to six different hospitals in Copenhagen. All strains were resistant to penicillin, streptomycin, and tetracyclines; the majority also to erythromycin. One strain was non-typable, all other strains belonged to phage group III.

All strains were resistant not only to methicillin but also to oxacillin, cloxacillin and cephalothin. A marked inoculum-size-effect was observed. With a large inoculum all strains grew in broth containing 500 or 1000 µg/ml of methicillin. In fluid medium a significant amount of

such strains to be significantly less resistant to methicillin than the parent penicillinase-producing strain.

The penicillinase negative strains had an "inherent" resistance to

some staphylococci of the "naturally occurring" type are actually, strains of *Staphylococcus aureus* characterized



previous treatment has been available, no patient in the material reported in this paper was treated with methicillin or with other penicillinase-resistant penicillins. Most strains of methicillin-resistant staphylococci reported by other authors have likewise been isolated from patients not treated with the new penicillins (10, 29). Actually, the first strains reported by *Jevons* (19) were isolated so soon after the introduction of methicillin and under such circumstances that it seems very probable that their occurrence had no relation whatever to the use of methicillin. Interesting is also the report by *Cetin & Ang* (9) on methicillin-resistant strains of *Staphylococcus aureus* isolated from patients in Turkey before methicillin had become available in that country.

It is, therefore, generally agreed that methicillin-resistant strains of *Staphylococcus aureus* isolated from clinical sources are "naturally resistant", but their ultimate mode of origin remains obscure (6).

It was shown in experiments reported above that penicillinase-negative strains derived from a methicillin-resistant *Staphylococcus aureus* were significantly less sensitive to penicillin G than other penicillinase-negative staphylococci. Thus, this staphylococcus is penicillin-resistant not only as a result of its production of penicillinase, but it has also an "inherent" resistance to penicillin G.

Strains with these properties may very well have existed before the introduction of methicillin, in as much as the existence of this kind of penicillin-resistance would have been extremely difficult, if not impossible, to demonstrate, at least in the routine laboratory where large numbers of staphylococci are tested.

Perhaps, it is justified to consider naturally occurring methicillin-resistant staphylococci as strains of *Staphylococcus aureus* characterized by high penicillinase-production and a certain degree of "inherent" resistance to penicillin. That such strains may occur without any relationship to the use of methicillin would not be surprising.

The incidence of methicillin-resistant strains of *Staphylococcus aureus* is, undoubtedly, increasing. *Jevons et al.* (20) found among 27,479 cultures 102 resistant strains giving an incidence of 0.37 per cent, but throughout the period January 1961 to September 1962 the incidence rose from less than 0.25 to 0.8 per cent. *Barber & Waterworth* (7) found that 3 per cent of the strains isolated from lesions in surgical patients were resistant. Endemic spread of methicillin-resistant staphylococci in a children's hospital has been reported by *Stewart & Holt* (29).

*Chabbert & Baudens* (10) found ten resistant strains among 82 strains received from February 1961 to February 1962. This very high incidence is possibly due to the fact that their material consisted of strains isolated from patients whose infections for various reasons did not respond to antibacterial chemotherapy. However, only one of their strains was isolated from a case treated with methicillin.

The findings summarized in Table 1 indicate that the incidence, also

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by high penicillinase-production associated with an "inherent" resistance to penicillin G

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## MATERIALS AND METHODS

*Normal Human Sera*

Sera from 187 unrelated adult healthy individuals from an earlier described material (Berg 1963) were used for association test. Sera from selected families of an earlier described family material (Berg & Mohr 1963) were used for linkage study.

*Anti Ag(a) Serum*

A sample of anti Ag(a) serum (C de B) was kindly provided by Dr B S Blumberg of the National Institutes of Health Bethesda Maryland. This is the original serum used by Allison and Blumberg when they detected the Ag system.

*Anti Lp(a) Serum*

Rabbit serum h 3 absorbed 2-5 with human serum of type Lp(a-) was used (see Berg 1963).

*Petri Dishes*

ALUMBAR dishes 7 cm in diameter were used.

*Glass Slides*

Washed and polished 5 X 5 cm glass slides (Menzel Glaser) were used.

*Agar*

For the Lp tests Difco Bacto Agar (Control No 45292a) was used (Difco Laboratories Detroit 1 Mich).

For the Ag tests Rhemagar (Behringwerke AG Marburg Op Nr 141) was used. The only reason for using different sorts of agar for the two systems was that one was more easily available than the other at the time of the investigations. For the Lp tests the first agar is just as good as the second and there is no reason to believe that this is not so even for the Ag tests.

*Double Diffusion Tests in Agar Gel*

For the Lp tests the agar gel was prepared in the Petri dishes as described elsewhere (Berg 1963). Six peripheral wells were cut around one central well the dia-

metr of the central well was 10 mm. Readings of precipitates were made after 2 days against a dark background by oblique illumination from below. Visible precipitate was registered as positive reaction, no visible precipitate as negative reaction.

For the Ag tests and all other experiments which included the anti Ag(a) serum the agar gel double diffusion experiments were performed on 5 X 5 cm glass slides.

To save anti Ag(a)

layer. The gel con-

cent (w/v) 1/15 M

On each slide 6 pc

distances between

serum was placed

and the precipitates were read after 2 days in the same manner as described for the Petri dishes used for the Lp tests.

*Photographic Registrations*

Photographic registrations of gel diffusion tests were made with a Leica 35 mm camera on Agfa Agfa film.

*Staining*

The precipitates in the agar gel double diffusion tests were stained according to Friel (1960) using either Oil Red O as a lipid stain or Amido Black 10 B as a protein stain (both from G T Gurr Ltd London).

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## COMPARATIVE STUDIES ON THE Lp AND Ag SERUM TYPE SYSTEMS

By

KÅRL BJØRG

Received 8 V 64

During recent years two different genetic systems within human serum  $\beta$ -lipoprotein have been described. The Ag system was described by Allison & Blumberg (1961). The authors demonstrated antigenic differences in human sera by means of an antibody in the serum from a patient who had received multiple transfusions, with Ouchterlony's agar gel double diffusion technique (Ouchterlony 1958). The antigen of the Ag system was shown to be  $\beta$ -lipoprotein (Blumberg, Dray & Robinson 1962).

The Lp system was discovered by Berg (1963). Using the same test technique, but with a heteroantisera, he found antigenic differences in human serum  $\beta$ -lipoprotein. The anti-Lp(a)-serum was obtained by intravenous immunization of rabbits with isolated human  $\beta$ -lipoprotein from one single donor. A subsequent absorption procedure was made for the demonstration of the type specificity of the antisera. Later studies have shown, however, that even whole human serum can be used for immunization. (These experiments will be described in another paper).

The genetics of the Lp system was studied in a material consisting of 94 Norwegian families with 305 children (Berg & Mohr 1963), and association and linkage studies were performed with the same material (Mohr & Berg 1963). Twenty-three normal human sera of known types within the Lp system were tested for the Ag(a) factor by Dr B. S. Blumberg of the National Institutes of Health, Bethesda, Maryland, and as far as these data extended, no interrelationship was found between the Ag(a) and the Lp(a) factors (see Berg 1963).

The purpose of the present paper is to present the results of more extensive comparative studies on the Lp and Ag serum type systems. The data presented are obtained from immunological studies, from association test in a material consisting of unrelated individuals, and from linkage test in an investigation of informative families.

intrabasin absorption technique. At first 15  $\mu$ l of the human serum for absorption was placed in one well. When this serum had diffused into the surrounding agar leaving the well empty 15  $\mu$ l of the anti serum was placed in the same well. At the same time the human sera against which the absorbed antisera were to be tested were placed in the other wells. The distribution of the different sera and the results obtained are shown in Fig. 2.

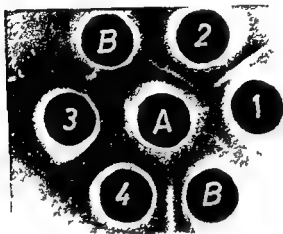


Fig. 2

Agar gel slide showing the results of intrabasin absorption experiments with anti Ag(a) and anti Lp(a) sera (see text)

The reagents (15  $\mu$ l of each) were

- A Human serum of type Lp(a<sup>+</sup>) Ag(a<sup>+</sup>)
- B Human serum of type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>)
- 1 Anti Ag(a) serum absorbed with human serum of type Lp(a<sup>-</sup>) Ag(a<sup>+</sup>)
- 2 Anti Ag(a) serum absorbed with human serum of type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>)
- 3 Anti Lp(a) serum absorbed with human serum of type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>)
- 4 Anti Lp(a) serum absorbed with human serum of type Lp(a<sup>-</sup>) Ag(a<sup>+</sup>)

Note that type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>) human serum has no reaction.

— u o v e i d

It was demonstrated that a normal human serum of type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>) was unable to absorb the anti Ag(a) antibody from the anti Ag(a) serum whereas a human serum of type Lp(a<sup>-</sup>) Ag(a<sup>+</sup>) absorbed the anti Ag(a) antibody completely. It was further demonstrated that a human serum of type Lp(a<sup>-</sup>) Ag(a<sup>+</sup>) was unable to absorb the anti Lp(a) antibody from the anti Lp(a) serum while a human serum of type Lp(a<sup>+</sup>) Ag(a<sup>-</sup>) absorbed all anti Lp(a) activity. These experiments seem to confirm that the Ag(a) and the Lp(a) factors are antigenically unrelated.

## EXPERIMENTS AND RESULTS

*Immunological Studies on the Lp(a) and Ag(a) Factors*

Using the anti-Lp(a)- and anti-Ag(a)-sera in agar gel double diffusion tests, human sera of all 4 possible reaction types were found. Thus some sera were found which were negative and others which were positive against both antisera, as well as sera of the reaction patterns Lp(a+) Ag(a—) and Lp(a—) Ag(a+) (See Fig 1)

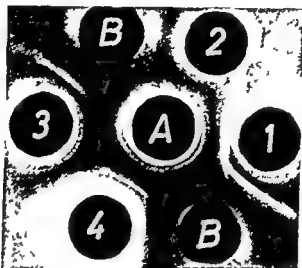


Fig 1

Agar gel slide demonstrating reactions between normal human sera (wells 1-4) and anti-Lp(a)-serum (wells B) and anti-Ag(a)-serum (well A). The precipitation pattern shows that the human sera are of the following phenotypes:

|        |        |        |
|--------|--------|--------|
| well 1 | Lp(a+) | Ag(a+) |
| well 2 | Lp(a—) | Ag(a—) |
| well 3 | Lp(a+) | Ag(a—) |
| well 4 | Lp(a—) | Ag(a+) |

Note reaction of non-identity between the Lp(a)- and Ag(a) precipitates near well 1

When human serum containing both the Ag(a) and the Lp(a) factors was tested simultaneously against anti-Lp(a)-serum and anti-Ag(a)-serum, a reaction of non-identity developed between the two precipitates (see Fig 1). The reaction of non-identity indicated that the Ag(a) and the Lp(a) factors are antigenically different.

The following absorption experiment was performed in order to investigate further the apparent difference between the Ag(a) and Lp(a) antigens.

Anti-Ag(a) serum was absorbed with a human serum of type Lp(a+) Ag(a—), and, for control, with a serum of type Lp(a—) Ag(a+). Likewise an anti-Lp(a)-serum was absorbed with a human serum of type Lp(a—) Ag(a+), and, for control, with a human serum of type Lp(a+) Ag(a—). To save the anti-Ag(a)-serum, these absorption experiments were performed on 5 × 5 cm agar slides, using an

I p(a+) Ag(a+) The antisera were placed such that they at the same time provided a control that the precipitation had really taken place in antibody excess. The arrangement on the agar slide and the result obtained is shown in Fig 3.

When human serum of type I p(a+) Ag(a+) was precipitated with excess of anti Lp(a) serum so that the human serum no longer reacted with anti Lp(a) serum in the neighbouring well the human serum still reacted strongly with anti Ag(a) serum in another neighbouring well. It was also found that when the human serum of type Lp(a+) Ag(a+) was precipitated with excess of anti Ag(a) serum so that it no longer reacted with anti Ag(a) serum it still reacted positively with anti Lp(a) serum. (See Fig 3).

This experiment seems to prove that the Lp(a) and Ag(a) factors refer to different  $\beta$  lipoprotein molecules.

### *Test for Association*

For the association test sera from 187 unrelated adult Norwegians already tested in the Lp system were tested with anti Ag(a) serum. Of these sera 62 (33.16 per cent) were of type I p(a+) and 125 (66.84 per cent) of type Lp(a-) whereas 72 (38.50 per cent) were of type Ag(a+) and 115 (61.50 per cent) of type Ag(a-).

The distribution of the individuals in this material with respect to the Lp(a) and Ag(a) factors is shown in Table 1.

TABLE 1

*Distribution of 187 Unrelated Individuals with Respect to the Lp and Ag Types*

|        | Ag(a+) | Ag(a-) | Total |
|--------|--------|--------|-------|
| Lp(a+) | 28     | 34     | 62    |
| Lp(a-) | 44     | 81     | 125   |
| Total  | 72     | 115    | 187   |

$$\chi^2 = 1.3415$$

1 D.F.

$$0.20 < p < 0.30$$

( $\chi^2$  for this table was calculated as described by Bradford Hill (1961) p. 172)

The result of the  $\chi^2$  test makes an association between the Lp(a) and Ag(a) factors highly improbable.

### *Test for Linkage*

For linkage test the method devised by Race & Sanger (1962) using "double back cross" families was applied. For this test one parent must be heterozygous for both the genes being investigated. As antisera are available against only one factor within each of the two systems under study the only way to select heterozygous parents is for each system to select the families of positive  $\times$  negative parents with at least one



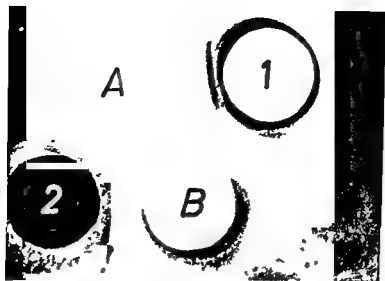


Fig 3

Tests in agar gel of human serum of type Lp(a+) Ag(a+) after precipitation with excess of anti-Ag(a)-serum and anti Lp(a)-serum respectively (see text)

The reagents (15  $\mu$ l of each) were

1 Human serum of type Lp(a+) Ag(a+) and anti-Ag(a)-serum

2 Human serum of type Lp(a+) Ag(a+) and anti-Lp(a) serum

A Anti-Lp(a)-serum

B Anti-Ag(a)-serum

The precipitation pattern shows that human serum of type Lp(a+) Ag(a+) still reacted with anti-Lp(a)-serum after precipitation with excess of anti-Ag(a)-serum, and with anti-Ag(a)-serum after precipitation with excess of anti Lp(a)-serum. This indicates that the Lp(a) and Ag(a) factors refer to different  $\beta$  lipoprotein molecules.

### *Attempt to Find whether the Lp(a) and Ag(a) Properties Belong to the Same Protein Molecules*

To investigate whether the Lp(a) and Ag(a) factors are located on the same lipoprotein molecules in persons possessing both factors, the following experiment was performed on a 5  $\times$  5 cm agar slide.

A human serum of type Lp(a+) Ag(a+) was precipitated with anti-Lp(a)-serum in antibody excess, and another sample of the same human serum with excess of anti-Ag(a)-serum. The anti-Lp(a)-serum used in this experiment was specifically absorbed with a Lp(a-) Ag(a-) human serum so that no additional amount of Ag(a) factor should be introduced through the human serum used for absorption of the anti-Lp(a)-serum. The anti-Ag(a)-serum was found to be of type Lp(a-).

At first, anti Ag(a)-serum was introduced into one well, and anti-Lp(a)-serum into another well, and the slide was placed in a moist chamber while the sera were diffusing into the agar gel. After some two hours the wells were completely empty, and now the human serum of type Lp(a+) Ag(a+) was placed in both the wells, and at the same time anti-Lp(a)-serum and anti-Ag(a)-serum were placed in two other wells to test the effect of the precipitation on the human serum of type

able (Berg & Vohr 1963), the informative families were selected in this way

Of the total of 41  $Lp(a+)$   $\times$   $Lp(a-)$  matings, 28 families had at least one  $Lp(a-)$  child. The parents of these 28 families were tested with respect to the  $Ag(a)$  factor. The children of the families in which one parent was  $Lp(a+) Ag(a+)$ , and the other  $Lp(a-) Ag(a-)$ , were tested with respect to the  $Ag(a)$  factor. Of 11 such families 4 families were found in which there was at least one  $Ag(a-)$  child. Because of the small chance to find double heterozygous individuals when antisera are available against only one factor within each of the two systems, the number of families informative for linkage studies, had to be small. The distribution of the  $Lp$  and  $Ag$  types in the four families informative as to possible linkage between the  $Lp(a)$  and the  $Ag(a)$  factors, is shown in Fig. 4.

Using the table of Race & Sanger (1962, p. 417), the linkage score,  $\lambda$ , and information\*,  $\gamma$ , for each family, and the  $\Sigma(\lambda)$  and  $\Sigma(\gamma)$  for the material of four families were found (Table 2).

TABLE 2

Linkage Scores ( $\lambda$ ) and Information ( $\gamma$ ) for 4 "Double Back Cross" Families with Respect to the  $Ag$  and  $Lp$  Systems

| Family No | Number of children in family | Ratio* | Linkage score $\lambda$ | Information $\gamma$  |
|-----------|------------------------------|--------|-------------------------|-----------------------|
| 1         | 3                            | 2:1    | -1                      | 3                     |
| 2         | 4                            | 2:2    | -2                      | 6                     |
| 3         | 3                            | 2:1    | -1                      | 3                     |
| 4         | 3                            | 2:1    | -1                      | 3                     |
|           |                              |        | $\Sigma(\lambda) = -5$  | $\Sigma(\gamma) = 15$ |

\* ratio = the ratio of children that would do for linkage to those that would then have to be recombinants (see Race & Sanger (1962) p. 417)

If there is linkage the  $\Sigma(\lambda)$  keeps on being positive as the data grow (Race & Sanger 1962). According to Race & Sanger (1962), the existence of linkage has been demonstrated at the 1 in 20 level of probability if  $\Sigma(\lambda)$  is greater than 1.64 |  $\Sigma(\gamma)$ , and at the 1 in 100 level if  $\Sigma(\lambda)$  is greater than 2.33 |  $\Sigma(\gamma)$ .

In the present material, the  $\Sigma(\lambda)$  was found to be -5 and the  $\Sigma(\gamma) = 15$  (Table 2). This value of  $\Sigma(\lambda)$  gives no suspicion of any linkage between the  $Lp$  and  $Ag$  systems.

The investigation of a larger number of informative families would be of great interest. In view of the small chance to find informative families, however, the family material would then have to be considerably extended. The material investigated till now must be regarded as a preliminary indication that no linkage exists between the  $Lp$  and  $Ag$  serum type systems.

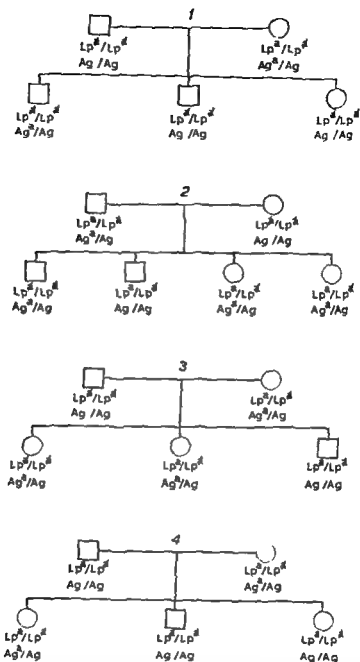


Fig. 4

Four "double back cross" families informative for linkage study between the Lp and Ag serum type systems of human  $\beta$  lipoprotein. Lp<sup>a</sup> is the designation for the recessive gene within the Lp system and Ag<sup>a</sup> for the recessive gene in the Ag system.

negative child. Consequently, a linkage study can only be made in a material of families where one of the parents is heterozygous for both the genes Lp<sup>a</sup> and Ag<sup>a</sup> and the other parent lacks both the genes, as the destination of the heterozygous parent's genes in the children is possible only in these families.

As a family material already tested for the Lp(a) factor was avail-

and the authors state that the slides should be washed and stained before reading. The genetic studies performed so far seem incomplete. As much as 96.7 per cent of white Americans reacted positively with this new antiserum and the authors found no American family where both the parents were negative. Only in a material of Micronesians they were able to find 4 families in which both parents were negative. The children of these families were all negative. The  $\beta$  lipoprotein factor detected with this new antiserum is called the Ag(b) factor. The relationship between the two genes determining the Ag(a) and Ag(b) factors is however not one of simple allelism.

So far comparative studies have only been performed with the well investigated Ag(a) factor. In the future similar studies should be performed with regard to the Ag(b) and other  $\beta$  lipoprotein factors which might be detected by means of isoantisera. The use of isoantisera does however present difficulties due to their rarity and because of the weakness of the antibody of some of the sera. Moreover frequently transfused patients often suffer from conditions prohibiting large blood donations. It is therefore reasonable to try to produce heteroantisera against the genetic groups of human serum  $\beta$  lipoprotein.

#### SUMMARY AND CONCLUSIONS

A comparative study of the Lp system as defined by absorbed rabbit immune serum anti Lp(a) serum and the Ag system as defined by anti Ag(a) serum of human serum  $\beta$  lipoprotein is presented.

Sera of all possible combinations of Lp and Ag types were found and no antigenic relationship between the Ag(a) and the Lp(a) factors was demonstrable.

It was shown that in sera possessing both the Lp(a) and Ag(a) factors these two factors referred to different molecules within the  $\beta$  lipoprotein.

From the experiments described it is obvious that even if the anti Ag(a) serum should contain several different antibodies none of them could be identical to that of the anti Lp(a) serum.

The investigation of sera from unrelated individuals gave no suggestion of any association between the Ag and Lp systems. A study of informative families gave no suggestion of linkage between the two systems.

It is therefore concluded that the Lp and Ag systems of human serum  $\beta$  lipoprotein are entirely independent and the necessity of a different nomenclature for the Lp and Ag systems is therefore confirmed by the present work.

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## DISCUSSION

It has earlier been demonstrated that both the Ag(a) and the Lp(a) factors belong to the human serum  $\beta$ -lipoprotein, and that the two factors are genetically determined (*Blumberg, Dray & Robinson 1962, Berg 1963*). It was therefore important to investigate whether any interdependency exists between the two systems.

The immunological tests showed that human sera of all combinations of Lp and Ag types exist, and that there is no antigenic relationship between the Lp(a) and Ag(a) factors. The reaction of non-identity between the Lp(a)- and Ag(a)-precipitates, the failing ability of a Lp(a—) Ag(a+) serum to absorb the antibody of an anti-Lp(a)-serum, and the failing ability of a Lp(a+) Ag(a—) serum to absorb the antibody of the anti-Ag(a)-serum are especially significant in this respect.

The experiment designed to study whether the Lp(a) and Ag(a) factors are located on the same  $\beta$ -lipoprotein molecule in persons possessing both factors, seems to prove that such individuals possess one kind of molecules with the Ag(a) but not the Lp(a) factor, and another kind of molecules with the Lp(a) and not the Ag(a) factor.

The testing of sera from unrelated individuals gave no evidence for any association between the Ag(a) and the Lp(a) factors.

The number of families informative for linkage studies between the Lp and Ag systems was small. As far as the available data extend, however, no evidence for linkage between the two systems was found.

It seems worth mentioning, that also a difference in the stability exists between the Lp(a) and Ag(a) factors. Fresh sera should be used for the Lp tests, as the reactions after some 6 months were considerably weaker and may even disappear within one year of storage in the frozen state (see *Berg 1963*). *Allison & Blumberg (1961)*, however, found that even 5 years old sera could be used for the tests for the Ag(a) factor.

The observations of the present study provide evidence that the Lp and Ag systems of human serum  $\beta$  lipoprotein are two independent systems concerning one serum protein. The ability of one serum protein to possess factors of two independent genetic systems, has previously been demonstrated for human 7S  $\gamma$  globulin. In this protein, factors belonging to both the Gm and Inv systems are located (*Harboe & Osterland 1963*), and although the Inv factors are also characteristics of other globulins, it seems reasonable to draw a parallel between this phenomenon and the Ag and Lp factors found in  $\beta$ -lipoprotein.

In addition to the anti-Ag(a) serum, *Blumberg & Riddell (1963)* have reported the detection of another serum from a patient who had received multiple transfusions, probably revealing another genetic factor of human serum  $\beta$ -lipoprotein. The reactions obtained with this anti-serum were weaker than those obtained with the anti-Ag(a)-serum,

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## PRECIPITATION REACTIONS IN AGAR GEL BETWEEN ALBUMIN AND $\beta$ LIPOPROTEIN OF HUMAN SERUM<sup>1</sup>

By

KÅRE BERG

Received 8 V 64

The agar gel double diffusion technique of Ouchterlony has been widely used in immunological studies (for review, see *Ouchterlony* 1962)

Several authors have stressed the multiplicity of factors that may influence the results of immunodiffusion tests in agar gel (for review, see *Crowle* 1960, 1961)

Precipitates in agar gel may appear, even if no antigen-antibody reaction takes place

Reactions between normal human serum and hemolysed erythrocytes (*Peeloom, Rose, Ruddy, Micheli & Grabar* 1960), and between human albumin and hemoglobin of human tissue extracts (*Melzgar & Grace* 1961), may give precipitates very similar to immune precipitates

Non immune precipitates in agar gel double diffusion tests have also been observed between normal serum and tissue extracts (*Melzgar & Grace* 1961, *Tomasi* 1961, *Berenbaum, Hitch & Cope* 1962) and between serum and extracts of tumours (*Deckers & Maisin* 1963) Recently, *Niece & Barrett* (1963) found that an antigen possessing enzymatic activity may provoke the formation of non immune precipitates in Ouchterlony plates

The purpose of the present paper is to describe another type of non-immune precipitates in agar gel double diffusion experiments, simulating antigen antibody reactions

It is known that  $\beta$  lipoprotein may interact with the agar medium, and a special type of agar, K agar, has been used for a quantitative precipitation test for human serum  $\beta$  lipoprotein (*Boyle & Moore* 1959) With ordinary laboratory grade agar, a circular precipitate may arise around the well containing serum  $\beta$  lipoprotein, especially if the agar is dissolved in barbital buffer (*Crowle* 1961) The utmost care is therefore necessary in performing agar gel diffusion tests with isolated  $\beta$ -lipoprotein

During the work on a new serum type system concerning human

<sup>1</sup> Aided by a grant from Dr A. Mithes Legat

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## PRECIPITATION REACTIONS IN AGAR GEL BETWEEN ALBUMIN AND $\beta$ LIPOPROTEIN OF HUMAN SERUM<sup>1</sup>

By

HÅRE BERG

Received 8 V 64

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<sup>1</sup> Aided by a grant from Dr A. Malthes Legat



$\beta$ -lipoprotein (the Lp system, *Berg* 1963), purified  $\beta$ -lipoprotein was tested against different immune sera. In the course of these experiments, the phenomenon to be described was first observed. It was found that isolated  $\beta$ -lipoprotein, when allowed to diffuse against different normal sera, led to the formation of distinct precipitates in the Ouchterlony dishes, thus representing a possibility for misinterpretation of agar gel tests including isolated  $\beta$ -lipoprotein. Further experiments showed that these non-immune precipitates depend upon an interaction between  $\beta$ -lipoprotein and albumin.

## MATERIALS AND METHODS

### *Human Sera*

A panel of 10 fresh normal human sera from laboratory personnel was used for the experiments. In one experiment, five sera, stored at  $-20^{\circ}\text{C}$  for 7 years, were also used.

### *Human $\beta$ -Lipoprotein.*

Fresh, undialysed normal human serum from single individuals was fractionated on a hydroxylapatite column with a stepwise elution technique according to *Hjerlen* (1959). Before use, all  $\beta$ -lipoprotein fractions from the chromatographic experiments were dialysed against a 0.85 per cent saline solution. The technique is described in detail elsewhere (*Berg* 1963).

To test the reproducibility of the chromatographic method, several batches of  $\beta$ -lipoprotein from two different donors, prepared with the standard chromatographic technique were tested, and all gave the phenomenon to be described. The  $\beta$ -lipoprotein fractions from 3 chromatographic runs with serum from one person were pooled, concentrated and used for the experiments to be described.

dry  
and  
volume of dialysate. This  $\beta$ -lipoprotein solution did not contain other serum proteins detectable by immunoelectrophoresis.

In addition to the  $\beta$ -lipoprotein prepared by chromatography, a sample of this lipoprotein prepared in the ultracentrifuge was used. This was Op-Nr 22263 from Behringwerke AG, Marburg<sup>1</sup>.

### *Solution of all Human Serum Proteins Except $\beta$ -Lipoprotein*

For the production of this solution, the two step fractionation technique of *Cramer* (1962) on the hydroxylapatite column was applied. By means of this technique all serum proteins except the  $\beta$ -lipoprotein were eluted from 8.5 ml of serum by 100 ml 0.25 M sodium phosphate buffer of pH 6.8. This eluate was concentrated to the same volume as that of the serum applied to the column. This solution did not react with specific rabbit-anti-human  $\beta$  lipoprotein-antiserum. It apparently contained all other serum proteins than  $\beta$  lipoprotein, when tested by immunoelectrophoresis.

### *Serum Lacking $\beta$ Lipoprotein*

A sample of serum from a patient with congenital absence of  $\beta$  lipoprotein was kindly supplied by Professor M Lamy Paris. This is patient number 1 in the publication of Lamy, Ficzal, Polonovski, Druet & Rey (1961).

### *Human Albumin*

Crystalline human albumin ("Reinste" Behringwerke AG Marburg Op-Nr 1916) was dissolved in a 0.85 per cent saline solution to obtain the following concen-

<sup>1</sup> I am indebted to Dr Heide and Dr Stürsko Behringwerke AG, Marburg for providing this sample.

trations (w/v) of albumin 4 per cent 2 per cent, 1 per cent, 0.1 per cent 0.01 per cent. When nothing else is particularly stated the 1 per cent solution was used. The albumin solutions did not contain other serum proteins detectable by immunoelectrophoresis.

#### *Human $\gamma$ Globulin*

A one per cent solution of human  $\gamma$  globulin prepared by chromatography on DEAE cellulose was kindly provided by Dr T. Reinskov, Institute of Forensic Medicine, University of Oslo. The  $\gamma$  globulin solution did not contain other serum proteins detectable by immunoelectrophoresis.

#### *Bovine Albumin*

Bovine albumin (30 per cent solution, Armour Pharmaceutical Company Ltd, Eastbourne, England) was diluted with 0.85 per cent saline to 6 per cent, 3 per cent, 1 per cent and 0.1 per cent solutions.

#### *Normal Rabbit Serum*

Serum from a healthy, not immunized 3 months old albino rabbit was used.

#### *Rabbit Anti Human Serum Antiserum*

Anti-Human serum (Behringwerke AG, Marburg, Op. Nr. 4706) was used as anti-serum in the immunoelectrophoretic tests of serum protein fractions.

#### *Specific Rabbit Anti Human $\beta$ Lipoprotein Antiserum*

The anti- $\beta$  lipoprotein sera were obtained by immunization of rabbits with isolated  $\beta$  lipoprotein using the immunization technique described by Berg (1963). When these sera were absorbed with serum from a patient with congenital absence of  $\beta$  lipoprotein (one part of the patient's serum to 20 parts of rabbit immune serum) they reacted only with the  $\beta$  lipoprotein and no other proteins of human serum.

#### *Specific Anti Lp(a) Antiserum from Rabbit*

Serum from rabbit A 3 absorbed in the proportion 2:5 with human serum of type Lp(a—) (Berg 1963) was used.

#### *Dialysis Bags*

Cellophane casings from Visking Co., Chicago, Ill. were used.

#### *Concentration of Chromatographic Fractions*

Fluted fractions from chromatographic columns were concentrated by dialysis against 0.1 M ethyleneglycol 20000 (Roche) as described by Kohn (1959).

#### *Petri Dishes*

ANMBR4 dishes 7 cm in diameter were used for agar gel double diffusion tests.

#### *Agar*

Difco Bacto Agar (Control No. 45292a) was used (Difco Laboratories, Detroit 1, Mich.).

#### *Agar Gel Double Diffusion Tests*

Each Petri dish was filled with 24 ml 1 per cent (w/v) agar containing 0.85 per cent (w/v) NaCl, 10 per cent (v/v) 1/15 M sodium phosphate buffer pH 7.0 and 1/10000 (w/v) merthiolate. This was the standard mixture for the agar dishes in the experiments to be described. The effect of the different reagents of the gel was further tested as described under Experiments and Results.

In the agar gel 6 peripheral wells were cut around one central well and the bottom of each well was sealed with a thin layer of agar. The diameter of each well was 4 mm and the distances between the circumferences of neighbouring wells were in all cases 5 mm. The reagents to be tested were distributed in the wells. The dishes were kept in a moist chamber at 37° C in the standard procedure. Readings of precipitates were made against a dark background by oblique illumination from below after 2, 3, 4, 6, 10, 12, 14, 24, 36 and 48 hours and after 3 and 7 days. All double diffusion experiments were made in duplicate. Some of the dishes were stained with Oil Red O as described by Uriel (1960).

Some dishes were stained for esterase activity, as described by Uriel (1961).

### *Immunoelectrophoresis*

Immunoelectrophoretic control of the purity of serum protein fractions were performed according to Grabar & Williams (1953) with the micromodification described by Scheidegger (1955).

### *Photographic Registrations*

Photographic registrations of gel diffusion and immunoelectrophoretic tests were made with a Leica 35 mm camera on Agepe Agfa film.

## EXPERIMENTS AND RESULTS

### *Occurrence of Precipitate between Isolated $\beta$ -Lipoprotein and Whole Human Serum*

When two neighbouring wells of a standard agar dish were filled with 60  $\mu$ l of whole human serum and 0.9 per cent  $\beta$ -lipoprotein solution respectively and the dish was incubated at 37° C, a precipitate became visible after approximately 3 hours. The precipitate was located about  $\frac{1}{3}$  way from the  $\beta$ -lipoprotein well to the well containing human serum. The precipitate was at first very sharp and dense, completely simulating a real immune precipitate (See Fig. 1). Some 5 hours after the start of the experiment, the precipitate became broader, less dense, and seemed to move somewhat in the direction of the  $\beta$ -lipoprotein well. The precipitate became steadily looser, and disappeared completely 10–12 hours after the start of the experiment.

These precipitates appeared against all the ten fresh human sera tested and also against all of 5 sera that had been stored for 7 years.

The change in density, and the subsequent disappearance of the precipitates, distinguished them clearly from true immune reactions, as for instance the reactions between human sera and a specific anti-Lp(a)-antiserum or anti- $\beta$ -lipoprotein-antiserum.

### *Identification of the Factor in Normal Whole Serum Reacting with Isolated $\beta$ -Lipoprotein*

As the precipitates only occurred between wells containing isolated  $\beta$ -lipoprotein and albumin or serum, and were not of a circular shape, it was concluded that they were not produced only through an interaction between  $\beta$ -lipoprotein and agar.

When a 1 per cent solution of human albumin was placed in the well next to the  $\beta$ -lipoprotein, it was found that this reagent produced a pre-

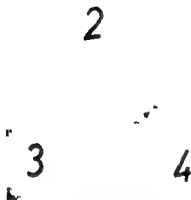


Fig 1

Precipitation in agar gel double diffusion test between isolated  $\beta$  lipoprotein and albumin of human serum

The reagents were

- 1 Saline solution 0.85 per cent
- 2 Whole human serum
- 3 Human albumin 1 per cent solution
- 4 Human  $\beta$  lipoprotein 0.9 per cent solution

Note reaction of identity between the two precipitates near the well containing human  $\beta$  lipoprotein

(The picture shows only the essential part of a standard agar dish)

precipitate against the 0.9 per cent  $\beta$  lipoprotein solution. This precipitate behaved in exactly the same manner as those obtained with whole human serum. When isolated  $\beta$  lipoprotein was tested simultaneously against whole human serum and isolated human albumin, a reaction of identity was formed between the two precipitates (Fig 1).

Isolated  $\beta$  lipoprotein was also tested against a 0.85 per cent saline solution, against another sample of isolated  $\beta$  lipoprotein from the same batch and against a 1 per cent solution of  $\gamma$ -globulin. No precipitate developed in any of these control experiments. Neither did any precipitate occur between pooled isolated albumin and the whole human serum used for isolation of the  $\beta$  lipoprotein, nor when this and other normal human sera were tested against each other.

To test if the precipitates could be produced with albumin from other species the  $\beta$  lipoprotein was also tested against bovine albumin and whole normal rabbit serum. A precipitate was obtained with both these reagents.

It was concluded that the factor in human serum precipitating  $\beta$  lipoprotein was albumin. It was further concluded that the  $\beta$  lipoprotein had to be isolated, at least from the albumin, for the occurrence of the precipitate while the albumin took part in the reaction, whether in the isolated state or in whole human serum. It was also concluded that not

In the agar gel 6 peripheral wells were cut around one central well and the bottom of each well was sealed with a thin layer of agar. The diameter of each well was 4 mm and the distances between the circumferences of neighbouring wells were in all cases 11 mm. The reagents to be tested were distributed in the wells. The dishes were kept in a moist chamber at 37° C, in the standard procedure. Readings of precipitates were made against a dark background by oblique illumination from below after 2, 3, 4, 5, 6, 10, 12, 14, 24, 36 and 48 hours and after 1 and 7 days. All double diffusion experiments were made in duplicate. Some of the dishes were stained with Oil Red O as described by Uriel (1960).

Some dishes were stained for esterase activity, as described by Uriel (1961).

### *Immunoelectrophoresis*

Immunoelectrophoretic control of the purity of serum protein fractions were performed according to Grabar & Williams (1953) with the micromodification described by Scheidegger (1955).

### *Photographic Registrations*

Photographic registrations of gel diffusion and immunoelectrophoretic tests were made with a Leica 35 mm camera on Agfa Agfa film.

## EXPERIMENTS AND RESULTS

### *Occurrence of Precipitate between Isolated $\beta$ -Lipoprotein and Whole Human Serum*

When two neighbouring wells of a standard agar dish were filled with 60  $\mu$ l of whole human serum and 1.9 per cent  $\beta$ -lipoprotein solution respectively and the dish was incubated at 37° C, a precipitate became visible after approximately 3 hours. The precipitate was located about  $\frac{1}{2}$  way from the  $\beta$ -lipoprotein well to the well containing human serum. The precipitate was at first very sharp and dense, completely simulating a real immune precipitate (See Fig 1). Some 5 hours after the start of the experiment, the precipitate became broader, less dense, and seemed to move somewhat in the direction of the  $\beta$ -lipoprotein well. The precipitate became steadily looser, and disappeared completely 10–12 hours after the start of the experiment.

These precipitates appeared against all the ten fresh human sera tested and also against all of 5 sera that had been stored for 7 years.

The change in density, and the subsequent disappearance of the precipitates, distinguished them clearly from true immune reactions, as for instance the reactions between human sera and a specific anti-Lp(a)-antiserum or anti- $\beta$ -lipoprotein-antiserum.

### *Identification of the Factor in Normal Whole Serum Reacting with Isolated $\beta$ -Lipoprotein*

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When a 1 per cent solution of human albumin was placed in the well next to the  $\beta$ -lipoprotein, it was found that this reagent produced a pre-

From the experiments with  $\beta$  lipoprotein and albumin in different concentrations it was found that while the 1 per cent albumin gave very distinct precipitates, the 0.1 per cent solution gave only a trace of precipitate occurring after 24 hours, and the 0.01 per cent solution gave no precipitate at all.

When the standard  $\beta$ -lipoprotein solution was diluted  $1/8$  it still gave a distinct precipitate, while the  $1/16$  dilution only gave a trace of a precipitate, visible after 24 hours.

Some of the dishes containing long lasting precipitates were washed for a few hours, dried, and stained with Oil Red O. The precipitates did not disappear during washing, and were stained red with this reagent demonstrating the presence of lipid in the precipitates.

When stained for esterase activity, the precipitates became red, indicating the presence of such activity.

In none of the experiments described were any precipitates visible after 36 hours or later. When the precipitates once had disappeared they never returned.

#### "Absorption" Experiment

An experiment to investigate if the precipitating ability could be removed from the albumin solution through "absorption" with  $\beta$  lipoprotein was made. One well was filled with the 0.9 per cent  $\beta$ -lipoprotein solution, and the dish was placed in a moist chamber until the solution had diffused into the surrounding agar gel. The well was then filled with 1 per cent albumin solution and a neighbouring well with 0.9 per cent  $\beta$  lipoprotein solution. No precipitate developed after the albumin solution had been submitted to this "intra basin absorption" with  $\beta$  lipoprotein before diffusing against the  $\beta$  lipoprotein of the neighbouring well. The phenomenon studied in this work thus behaves like true immune precipitation, also with respect to the effect of "absorption".

#### Effect of Salt Concentration in the Agar Gel on Albumin $\beta$ -Lipoprotein Precipitate Formation

To test the effect of the salt concentration of the agar gel, an experiment was performed, in which the concentration of NaCl in the gel was varied, all other conditions being kept constant. The different concentrations of NaCl are listed in Table 3.

The diffusing reagents were 1 per cent albumin solution and 0.9 per cent  $\beta$  lipoprotein solution. The concentration of 2 per cent or higher did not prevent the development of true immune precipitates.

only human albumin, but also bovine and probably also rabbit albumin, could precipitate human  $\beta$ -lipoprotein

### *Quantitative Studies of the Interaction between Albumin and $\beta$ -Lipoprotein*

To test the effect of the amount of the two reagents on the development of precipitate, different dilutions of the 0.9 per cent  $\beta$ -lipoprotein solution were tested against a 1 per cent solution of human albumin, and the 0.9 per cent solution of  $\beta$ -lipoprotein was tested against different concentrations of human and bovine albumin. The results are shown in Tables 1 and 2

TABLE 1

*Reaction Pattern by Double Diffusion in Agar Gel between Human Albumin and  $\beta$ -Lipoprotein*

| Human serum Albumin | Dilutions of a 0.9 per cent $\beta$ lipoprotein solution |     |     |     |      |      |
|---------------------|--|-----|-----|-----|------|------|
|                     | 1:1  | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| 1 per cent solution | +  | +   | +   | (+) | ±    | —    |

+ = distinct precipitate

(+) = weak precipitate

± = very weak (and late occurring) precipitate

— = no visible precipitate

TABLE 2

*Reaction Pattern by Double Diffusion in Agar Gel between Human  $\beta$  Lipoprotein and Albumin*

| Human $\beta$ lipoprotein solution | Concentrations of solutions of albumin |    |    |      |       |    |        |    |      |
|------------------------------------|--|----|----|------|-------|----|--------|----|------|
|                                    | Human                                  |    |    |      |       |    | Bovine |    |      |
|                                    | 4%                                     | 2% | 1% | 0.1% | 0.01% | 0% | 3%     | 1% | 0.1% |
| 0.9 per cent                       | +                                      | +  | +  | ±    |       | +  | +      | +  | —    |

For significance of +, ± and — see Table 1

It was found that while 1 per cent albumin gave precipitates behaving very like those obtained with whole human serum, the stronger albumin solutions had a tendency to give more long-lasting precipitates. This was particularly obvious when the 4 per cent and 2 per cent human albumin and the 6 per cent bovine albumin solutions were used. With these reagents the precipitates were visible even after 24 hours. Whole serum from a patient with congenital absence of  $\beta$ -lipoprotein and the fraction from the hydroxylapatite chromatography of human serum eluted with 0.25 M phosphate buffer with a pH of 6.8 behaved very similar to the stronger albumin solutions, with more long-lasting precipitates.

From the experiments with  $\beta$  lipoprotein and albumin in different concentrations it was found that while the 1 per cent albumin gave very distinct precipitates the 0.1 per cent solution gave only a trace of a precipitate occurring after 24 hours and the 0.01 per cent solution gave no precipitate at all.

When the standard  $\beta$  lipoprotein solution was diluted  $\frac{1}{4}$  it still gave a distinct precipitate while the  $\frac{1}{16}$  dilution only gave a trace of a precipitate visible after 24 hours.

Some of the dishes containing long lasting precipitates were washed for a few hours, dried and stained with Oil Red O. The precipitates did not disappear during washing and were stained red with this reagent demonstrating the presence of lipid in the precipitates.

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The diffusing reagents were 1 per cent albumin and 0.9 per cent  $\beta$  lipoprotein. The gel was incubated at 2 per cent or higher did not prevent the development of true immune precipitates.



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To test the effect of the amount of the two reagents on the development of precipitate, different dilutions of the 0.9 per cent  $\beta$ -lipoprotein solution were tested against a 1 per cent solution of human albumin, and the 0.9 per cent solution of  $\beta$ -lipoprotein was tested against different concentrations of human and bovine albumin. The results are shown in Tables 1 and 2.

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|---------------------|--|-----|-----|-----|-------|------|
|                     | 1:1  | 1:2 | 1:4 | 1:8 | 1:16  | 1:32 |
| 1 per cent solution | +  | +   | +   | (+) | $\pm$ | —    |

- + = distinct precipitate  
 (+) = weak precipitate  
 $\pm$  = very weak (and late occurring) precipitate  
 — = no visible precipitate

TABLE 2

*Reaction Pattern by Double Diffusion in Agar Gel between Human  $\beta$  Lipoprotein and Albumin*

| Human $\beta$ lipoprotein solution | Concentrations of solutions of albumin |    |    |       |       |        |    |    |      |  |
|------------------------------------|--|----|----|-------|-------|--------|----|----|------|--|
|                                    | Human                                  |    |    |       |       | Bovine |    |    |      |  |
|                                    | 4%                                     | 2% | 1% | 0.1%  | 0.01% | 6%     | 3% | 1% | 0.1% |  |
| 0.9 per cent                       | +                                      | +  | +  | $\pm$ |       | +      | +  | +  | —    |  |

For significance of +  $\pm$  and — see Table 1

It was found that while 1 per cent albumin gave precipitates behaving very like those obtained with whole human serum, the stronger albumin solutions had a tendency to give more long lasting precipitates. This was particularly obvious when the 4 per cent and 2 per cent human albumin and the 6 per cent bovine albumin solutions were used. With these reagents the precipitates were visible even after 24 hours. Whole serum from a patient with congenital absence of  $\beta$ -lipoprotein and the fraction from the hydroxylapatite chromatography of human serum eluted with 0.25 M  $\beta$ -phosphate buffer with a pH of 6.8, behaved very similar to the stronger albumin solutions, with more long-lasting precipitates.

all the other pH values 1/15 M phosphate buffers were used. Dishes with the following pH values were prepared: 5.0, 6.0, 7.0, 8.0 and 8.6.

It was found that the precipitate was most distinct at pH 7.0 but was also recognizable at pH 8.0 and 8.6 although it developed a little later and appeared less dense. All these precipitates disappeared after 10–12 hours.

At pH 5.0 and 6.0 circular precipitates around the well containing  $\beta$  lipoprotein developed which probably could mask a precipitate between albumin and  $\beta$  lipoprotein.

#### *Effect of Temperature on the Albumin $\beta$ Lipoprotein Precipitate Formation*

All experiments hitherto described were performed at 37° C. To test the effect of temperature, standard agar dishes were kept at 4°, 20° and 37° C. The albumin  $\beta$  lipoprotein precipitate developed at all three temperatures, it was gone after 12 hours at 37° C, at this time still present at 20° C and 4° C, and after 24 hours still distinct at 4° C and also present at 20° C. All precipitates had vanished after 36 hours.

#### *Experiment with $\beta$ Lipoprotein Prepared in the Ultracentrifuge*

To investigate whether the precipitates described only occurred with  $\beta$  lipoprotein prepared by hydroxylapatite column chromatography, a 0.5 per cent solution of  $\beta$  lipoprotein prepared in the ultracentrifuge was tested against 1 and 2 per cent solutions of human albumin, and against 1 and 6 per cent solutions of bovine albumin. A precipitate occurred between the  $\beta$  lipoprotein and each of the albumin solutions mentioned.

### DISCUSSION

The experiments described demonstrate that a precipitate in agar gel can occur between albumin and  $\beta$  lipoprotein of normal human serum. From this study it seems necessary for the  $\beta$  lipoprotein to be in the isolated state to give the precipitate described whereas the albumin forms precipitate whether isolated or present in whole serum. It is at present not known if sera exist in which the  $\beta$  lipoprotein behaves as if it were in the isolated state, or sera which for some reason make  $\beta$  lipoprotein of other sera behave as if it were in the isolated state, thus giving precipitates between whole human sera. This author has, for instance, not had the opportunity to study sera from patients with analbuminemia.

The precipitate between albumin and  $\beta$  lipoprotein is very like a true immune precipitate, also as to the effect of "absorption". It is, however, clearly distinguishable from real immune precipitates at least with the composition of the agar gel used as the standard in the present work,

TABLE 3

*Reaction Pattern by Double Diffusion in Agar Gel between  $\beta$  Lipoprotein and Albumin of Human Serum at Different Concentrations of NaCl in Agar Gel*

| Reacting solutions              | Concentrations of NaCl in agar gel |      |      |    |    |    |     |
|---------------------------------|------------------------------------|------|------|----|----|----|-----|
|                                 | 0%                                 | 0.4% | 0.9% | 2% | 3% | 5% | 10% |
| Albumin and $\beta$ lipoprotein | +                                  | +    | +    | —  | —  | —  | —   |

For significance of + and —, see Table 1

This gel contained 1 per cent (w/v) agar, 0.07 M sodium phosphate buffer pH 7.0 and 1/10000 (w/v) merthiolate. In agar dishes of this composition, the precipitate between albumin and  $\beta$ -lipoprotein tended to last much longer than in the experiments earlier described in this paragraph. The precipitates were distinct even after 24 hours.

#### *Effect of the Concentration of Agar in the Gel on the Albumin- $\beta$ -Lipoprotein Precipitate Formation*

Agar dishes with different concentrations of agar, as shown in Table 4, were prepared, all other conditions being kept as described for the standard dish.

TABLE 4

*Reaction Pattern by Double Diffusion in Agar Gel between  $\beta$  Lipoprotein and Albumin of Human Serum at Different Concentrations of Agar in Gel*

| Reacting solutions              | Concentrations of agar in gel |    |      |    |    |    |
|---------------------------------|-------------------------------|----|------|----|----|----|
|                                 | 0.5%                          | 1% | 1.5% | 2% | 3% | 5% |
| Albumin and $\beta$ lipoprotein | +                             | +  | +    | +  | —  | —  |

For significance of + and — see Table 1

It was found, that when the agar concentration was 3 per cent or more, no precipitate between albumin and  $\beta$ -lipoprotein was formed. When the agar concentration was 1.5 per cent, the precipitate developed after 5 hours, and when the concentration was 2 per cent, after about 7 hours. When the concentration was 0.5 per cent, the precipitate could still be observed after 24 hours. At all other agar concentrations tested, it disappeared 10–12 hours after the experiments were started.

#### *Effect of pH on the Albumin- $\beta$ Lipoprotein Precipitate Formation*

In agar dishes of otherwise standard composition, the pH was varied by the addition of buffers of different pH values. The volume of the buffer was as given in the standard description. The buffer used to obtain a pH of 8.6, was barbital buffer with an ionic strength of 0.1. For

all the other pH values 1/15 M phosphate buffers were used. Dishes with the following pH values were prepared 5.0, 6.0, 7.0, 8.0 and 8.6.

It was found that the precipitate was most distinct at pH 7.0 but was also recognizable at pH 8.0 and 8.6 although it developed a little later and appeared less dense. All these precipitates disappeared after 10-12 hours.

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and when the dishes are kept at 37° C. The precipitates are transient, appearing about 3 hours after the start of the experiment, and usually disappearing 7-9 hours later. They are thus easily distinguishable from the Lp(a) precipitates (Berg 1963), which appear at a time when the albumin- $\beta$ -lipoprotein precipitates have dissolved. The formation of an albumin- $\beta$ -lipoprotein precipitate is influenced by the composition of the agar gel. The most important finding in this connection probably is that a NaCl concentration of 2 per cent or more in the gel completely prevents the formation of the described precipitates, but does not prevent the development of true immune precipitates. This gives a practical method to control if a precipitate including human  $\beta$ -lipoprotein represents a true immunological reaction or not.

The true nature of the precipitation observed between albumin and  $\beta$ -lipoprotein is, at present, unknown.

From the knowledge of the multiple substances, that may precipitate lipoproteins when techniques other than gel diffusion are applied, (Amenta & Waters 1960, Cornwell & Kruger 1961), a transient complex formation between albumin and  $\beta$  lipoprotein under certain conditions, seems possible. The inhibition of precipitate formation when the salt concentration is raised, is consistent with the findings of Berenbaum, Kitch & Cope (1962). They concluded that the non-immune precipitates they observed, therefore involved a salt like combination. Tomasi (1961) also found that his non-immune precipitates were inhibited when the ionic strength was increased, and concluded that electrostatic factors were important for the reaction. From the findings in the present work, it is possible that these conclusions of Tomasi (1961) and Berenbaum, Kitch & Cope (1962) also are valid for the albumin  $\beta$ -lipoprotein precipitation.

It is known that albumin has the ability to form non-immune precipitates in agar gel (Metzgar & Grace 1961). The present study shows that also  $\beta$ -lipoprotein can take part in the formation of such precipitates. It remains to be investigated if any of the earlier reported non immune precipitates are related to the phenomenon described in this paper. As to the findings of precipitates between albumin and tissue extracts, it should be mentioned that such extracts may contain considerable amounts of serum proteins, and that  $\beta$ -lipoprotein then, to some extent at least, perhaps could take part in the formation of these non-immune precipitates.

The detection of 2 genetic systems within the  $\beta$ -lipoprotein recently (Allison & Blumberg 1961, Berg 1963) will probably lead to extensive immunological studies of this protein. It is necessary to have in mind the phenomenon described in this paper in the study of isolated  $\beta$ -lipoprotein by means of double diffusion in agar gel.

The finding of a precipitate in gel diffusion tests does not prove that an antigen-antibody reaction has taken place. The possibility that a precipitate is non-immune, should be considered when interpreting the

results of such experiments. Experiments to control if a precipitate really represents an antigen antibody reaction should always be performed.

## SUMMARY AND CONCLUSIONS

- 1) A precipitate formation in agar gel between  $\beta$  lipoprotein and albumin very like true immune precipitate, is reported.
- 2) So far, the reaction has only been observed when  $\beta$ -lipoprotein is present in the isolated state, whereas albumin can either be isolated or present as a constituent of whole serum.
- 3) In the author's composition of the agar gel, the precipitate is transient, and therefore easily distinguishable from true immune precipitates, when the experiments are performed at  $37^{\circ}\text{C}$ . The precipitate may be more long-lasting under other circumstances, and thereby represent a possibility for false interpretation of agar gel double diffusion tests.
- 4) The presence of NaCl in the gel, inhibits the precipitate formation, a concentration of 2 per cent or more prevents it completely.
- 5) The necessity is stressed, not to interpret a precipitation in agar gel as an immunological reaction before the possibility of a non immune precipitate is excluded.

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## BRIEF REPORT

THE EFFECT OF DIMETHYLGLYCINE (PENICILLAMINE) ON THE MOBILITY OF  $\beta_{1A}$  GLOBULIN IN HUMAN SERUM A PRELIMINARY REPORT

By B. Mansa

$\beta_{1C}$  globulin is one of the human serum components which is well characterized by immunoelectrophoresis (3). Some relationship of this protein with the complement system is to be expected due to its transformation to  $\beta_{1A}$  globulin which is demonstrable after prolonged storage of sera (6). Muller Eberhard *et al.* (6) have described the isolation of the two globulins in question and have characterized them with respect to electrophoretic mobilities and sedimentation coefficients. In addition

cillamine) is presented

Pathological sera were treated with 0.2 M penicillamine at room temperature for two hours at pH 7.0-7.5 prior to immunoelectrophoretic analyses. Using polyvalent rabbit antisera against human serum some of the treated sera were found to develop a precipitation line within the intermediate mobility range for  $\gamma$  globulins. For further analysis

including

used. With these

unknown components

antiserum

$\beta_{1A}$  globulin and the unknown component were demonstrated as two individual lines.

After penicillamine treatment it was assumed that there was a correlation with the amount of  $\beta_{1A}$  globulin present in the individual samples. Preliminary

Fresh human serum from a healthy individual containing hydrazine in a final concentration of 0.1% (pH 7.38) suffered saline (pH 7.38) the sample was subjected to immunoelectrophoretic analysis of the

reduced sample demonstrated the presence of the unknown component. Furthermore the analyses showed the expected conversion of  $\beta_{1C}$  globulin to  $\beta_{1A}$  globulin as a result of hydrazine treatment. In a control experiment in which incubation with saline was substituted for the hydrazine treatment it was impossible to demon-

strated

Received 16 vii 64 from the Department of Biophysics, Statens Serum Institut, Copenhagen.

<sup>1</sup> Commercially available from Centraal Laboratorium van de Dienst Het Nederlandsche Roodkruis, Amsterdam.

<sup>2</sup> Rabbit anti-F<sub>1C</sub> rabbit C<sub>1</sub> human

disposal through the courtesy of prof. P. Klein, Mainz.



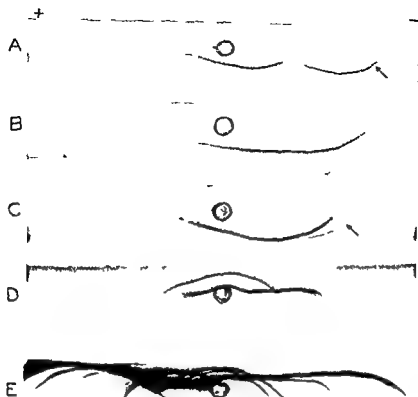


Fig. 1 illustrates the immunoelectrophoretic demonstration of the new component  $\gamma_{511}$  globulin. A stored serum sample from a patient with pleuropneumonia which contained  $\beta_{1\lambda}$  globulin was electrophoretically separated after penicillamine treatment (at A, B and C). The same untreated serum was separated electrophoretically as a control (at D and F). The following antisera were used: I anti  $C_{2C4}$ ; II anti  $\gamma_1$  macroglobulin; C and D a mixture of 3 volumes anti  $C_{2C4}$  and 2 volumes anti  $\gamma_{1\lambda}$  globulin; F a polyvalent anti human serum. The serum sample is seen to contain  $\beta_{1\lambda}$ - and  $\beta_{1C}$  globulin when analysed with the anti  $C_{2C4}$ . The new component  $\gamma_{511}$ -globulin was demonstrable within the intermediate  $\gamma$  globulin mobility range after the penicillamine treatment. The precipitation line of this component is indicated by arrows in Fig. 1 A and C. [For further details see text.]

line corresponded to the  $\beta_{1E}$  globulin line developed with the above mentioned anti  $C_{2C4}$  antiserum. This result indicates that the unknown component is immunologically related to  $\beta_{1\lambda/C}$  globulin. Stored serum samples in which part of the  $\beta_{1C}$  globulin remained unchanged showed after penicillamine treatment a three humped precipitation line the individual parts of which consisted of 1)  $\beta_{1\lambda}$  globulin, 2)  $\beta_{1C}$ -globulin and 3) the unknown component. The identification of these 3 lines was established by immunoelectrophoretic analysis with the anti  $C_{2C4}$  antiserum in the antibody trough. A partial fusion of the lines presented evidence for the immunological relationship between  $\beta_{1C}$  globulin and the unknown component.

The chemical nature of the action of penicillamine on  $\beta_{1\lambda}$  globulin is unknown but presumably a reduction of disulphide bonds takes place. The change in net charge demonstrated by the decrease in the electrophoretic mobility can probably be explained by assuming that a heavily charged but rather small part of each molecule is split off from the main part and that only the major part of the molecule is demonstrable by the technique used.

It is proposed that the unknown component be named  $\gamma_{511}$  globulin. Such a name would give an indication of its mobility range would also show its relationship to

the complement component  $\beta_{1C}$  globulin ( $C_{3a}$ ) and finally point out that it appears after treatment with a S-S splitting agent

**Summary** The present study has shown that  $\beta_{1A}$  globulin in human serum is sensitive to the disulphide splitting agent penicillamine. This treatment resulted in a considerable decrease in electrophoretic mobility. The split product was demonstrable within the intermediate  $\gamma$  globulin mobility range by immunoelectrophoretic analysis. Identical results were obtained by treatment of  $\beta_{1A}$  globulin in stored sera and in hydrazine treated sera. The new component could not be demonstrated after penicillamine treatment of  $\beta_{1C}$  globulin in fresh serum. An immunological relationship to  $\beta_{1A}/C$  globulin was established.  $\gamma_{5H}$  globulin is proposed as a name for this new component.

**References** 1 Burtin P. *Rev franç Etud clin biol* 6: 284, 1961—2 Ellis H. 4 & Gell P. G. H. *Nature* 181: 1667, 1958—3 Grabar P. & Burtin P. *Analyse immuno-electrophoretique*. Masson, Paris, 1960—4 Muller Eberhard H. J. Isolation and description of proteins related to the human complement system. *Diss. Almquist & Wiksell*, Uppsala, 1961—5 Muller Eberhard H. J. & Biro C. *Protides of the Biological Fluids*, Proc. 11th Colloquium, Bruges, 1963. Amsterdam, 1964, p. 420—6 Muller Eberhard H. J., Nilsson L. & Ironsson T. *J exp Med* 111: 201, 1960—7 Ouchterlony O. In P. Hallöe & B. H. Waksman (Eds.) *Progress in Allergy*, 6: 30, 1962—8 Taylor A. W. & Leon W. A. *J Immunol* 87: 284, 1959

*Acta path et microbiol scandinav* 62: 301-302, 1964

## BRIEF REPORT

ISOLATION OF THE TRIC AGENT FROM 3 CASES OF NEONATAL INCLUSION  
HISTORIOGRAPHY AND FROM THE CERVIX OF ONE OF THE MOTHERS

By C. H. Nordhorst

The Tric agent  
by  
in  
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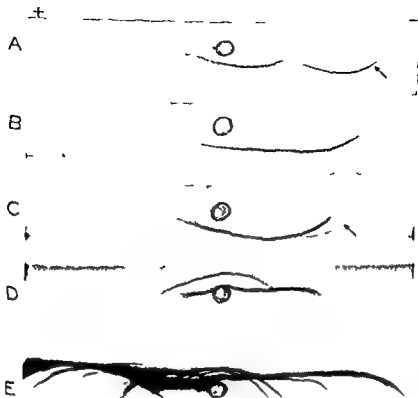


Fig 1 illustrates the immunoelectrophoretic demonstration of the new component  $\gamma_{5H}$  globulin. A stored serum sample from a patient with pleuropneumonia which contained  $\beta_{1A}$ -globulin was electrophoretically separated after penicillamine treatment (at A, B and C). The same untreated serum was separated electrophoretically as a control (at D and F). The following antisera were used: A anti  $C_{\gamma 4}$ ; B anti  $\gamma_1$ -macroglobulin L and D a mixture of 3 volumes anti  $C_{\gamma 4}$  and 2 volumes anti  $\gamma_1$  globulin. F a polyvalent anti human serum. The serum sample is seen to contain  $\beta_{1A}$  and  $\beta_{1C}$  globulin when analysed with the anti  $C_{\gamma 4}$ . The new component  $\gamma_{5H}$  globulin was demonstrable within the intermediate  $\gamma$  globulin mobility range after the penicillamine treatment. The precipitation line of this component is indicated by arrows in Fig 1 A and C. For further details see text.

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It is proposed that the unknown component be named  $\gamma_{5H}$  globulin. Such a name would give an indication of its mobility range, would also show its relationship to

## BRIEF REPORT

## IDENTIFICATION OF CANDIDA ALBICANS

By A Stenderup and J Brown Thomsen

Various methods for the quick identification of *Candida albicans* have been published during recent years. In 1960 *Taschdjian et al* published the filamentation test performed in serum and later *Buckley & van Uden* (1963) modified the test using eggwhite instead of serum claiming that eggwhite could be used on slides as well as in tubes. They also indicated that the medium on which the yeasts had been cultured might be of significance.

*Rosenthal & Furnari* (1958) used slide agglutination in the identification of *Candida albicans* using specific antiserum, which is now commercially available (Difco).

A "clotting" reaction was described by *Kaminsky & Quinlan* in 1963.

As we have not been able to verify the so called "clotting" reaction, we used different methods we have made a comparison of the results obtained with the different methods. We used 196 other strains of *Candida albicans* 107, torulopsis 1

used in this laboratory (Brain Heart  
yein and penicillin, Sabouraud agar  
significance for the results obtained

The results obtained when we used the different variations in the filamentation test are given below.

*Filamentation Test*

|                            | <i>Candida albicans</i> (107 strains) |          |
|----------------------------|---------------------------------------|----------|
|                            | positive                              | negative |
| Eggwhite (slide technique) | 26 (24%)                              | 81 (76%) |
| Eggwhite (tube technique)  | 107 (100%)                            | 0        |
| Serum (tube technique)     | 107 (100%)                            | 0        |

All other strains (397) showed negative reactions

Results of slide agglutination test are shown below

*Candida albicans* antiserum

|                             | Reactions |    |    |     | % positive |
|-----------------------------|-----------|----|----|-----|------------|
|                             | (+)       | +  | ++ | +++ |            |
| <i>C. albicans</i> (107)    | 3         | 20 | 70 | 14  | 100%       |
| <i>C. tropicalis</i> (30)   | 1         | 8  | 3  | 0   | 40%        |
| <i>C. parapsilosis</i> (38) | 0         | 0  | 2  | 0   | 5%         |

All other strains showed negative reactions

We have not been able to verify the so called "clotting" reaction, which we found negative in all strains but one

Received 20 vii 64 from the Institute of General Pathology and Bacteriology,  
University of Aarhus (Heads professor E. A. Freundt and professor A. Stenderup)  
Aided by a grant from Statens almindelige Videnskabsfond

A typical clinical picture of inclusion blennorrhoea was seen in all five babies examined. Repeated conjunctival scrapings revealed in each case a number of cytoplasmic inclusion bodies in epithelial cells. Positive isolates were obtained from the conjunctiva of 3 of the 5 babies.

Cervical scrapings were obtained from three of the five mothers with inclusion positive babies. These smears disclosed a cytology very much similar to that of the conjunctiva after infection with the TRIC agent but the presence of inclusion bodies could not be demonstrated. A TRIC isolate was however obtained from one of the three mothers. This woman was the mother of one of the three babies who yielded a positive isolation.

From each positive patient at least 2 isolates were obtained from specimens collected on consecutive days. The strains were usually recognized in the second yolk sac passage and the embryo was killed in this or the third passage. The impression smears of the yolk sac membranes disclosed numerous free elementary bodies. In morphology and colour these elementary bodies were found to resemble those of other members of the psittacosis LGV trachoma group of agents. The egg LD<sub>50</sub> of the strains after establishing was of the order to 10<sup>4.5</sup> to 10<sup>6.5</sup> per ml. All strains have by now been through 7 to 25 egg passages.

During the same period as the above mentioned isolations were made about 20 young women were examined for a TRIC agent infection of the genital tract. These patients were selected among patients attending a venereal disease clinic because their sera fixed complement to a titer of 1:10 to 1:40 with a LGV antigen. Cervical scrapings from these patients did not indicate a TRIC infection and repeated attempts to isolate an agent from these 20 patients have so far been negative.

Antigen prepared from the isolates by the method described by Valkert & Møller Christensen (6) fixed complement with LGV antisera to full titer. Strains from the conjunctiva as well as the strain from the cervix inoculated onto the conjunctival sac of monkeys (*Macaca cynomolgus*) res.

intraeytoplasmatic inclusion bodies

eyes. The clinical course in the monkey

derived from the conjunctiva or from the cervix (7).

One of the strains isolated from the conjunctiva of a child produced by accident a typical picture of inclusion conjunctivitis in a laboratory worker. A number of inclusion bodies was found in conjunctival smears from the affected eye and a TRIC strain was isolated in eggs.

**Summary.** From 3 out of 5 active cases of neonatal inclusion blennorrhoea in Denmark an agent belonging to the trachoma inclusion conjunctivitis (TRIC) group has been isolated by the chick embryo technique. A similar agent was isolated from the cervix of one of the three mothers.

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# A STUDY OF EPIDERMAL TUMOURIGENESIS IN THE HAIRLESS MOUSE WITH SINGLE AND WITH REPEATED APPLICATIONS OF 3 METHYLCHOLANTHRENE AT DIFFERENT DOSAGES

By

OJAV HILMAR IVERSEN and ULLA IVERSEN

Received 12 III 64

A sufficiently large single dose of a carcinogenic hydrocarbon, administered as a surface application, can provoke papillomas, and even carcinomas, in mouse skin (see, for instance, *Findlay 1925, Uder & Norton 1939, Law 1941, Cramer & Slowell 1943, Engelbreth-Holm & S Iversen 1951, Andreassen & Engelbreth Holm 1953, Rorum 1954, Klein 1956, Roe 1956, Poel 1959 and Terracini, Shubik & Della Porta 1960*).

The last mentioned authors concluded from their experiments that a critical dose level exists at which single application of carcinogens becomes fully effective. One of us (*Iversen 1962*) has demonstrated that when single applications of methylcholanthrene at different dosages were used the tetrazolium test for carcinogenicity revealed a "step" between the 1/32 and 1/16 per cent solutions. The test was negative with solutions of 1/32 per cent and lower, whereas it was positive at concentrations of 1/16 per cent and higher.

The present study was undertaken (i) to see if the critical dose level demonstrated for the action of DMBA on Swiss mice by *Terracini, Shubik & Della Porta* could also be observed for methylcholanthrene on hairless mice (ii) to see if a possible critical dose level, as measured by the tumour yield, might be observed at the same dose level as the step in the tetrazolium test, and (iii) to observe if there is some characteristic difference between the results when single and when repeated doses are administered.

## MATERIALS AND METHODS

A total of 330 hairless mice of the strain hr/hr were used. The animals were housed in wooden cages and fed our standard diet. The —

From this it must be concluded that the filamentation test is a quick and reliable test in the identification of *Candida albicans* when the reaction is performed in tubes using either serum or eggwhite. The slide technic with eggwhite is unreliable.

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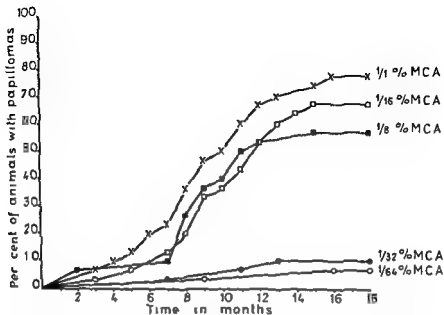


Fig 1

Percentage of tumourbearing animals during the observation period after one single painting with methylcholanthrene in benzene at different concentrations

b) *Single application of methylcholanthrene* The results are presented in Table 2 and in Fig 1. It is seen that whereas the two lowest dosages viz the 1/64 and the 1/32 per cent provoked very few papillomas in a low percentage of the mice the three higher dosages produced many papillomas in many of the animals. Evidently, the dose/response curve shows a pronounced increase between the two dosages 1/32 and 1/16 per cent corresponding to a borderline application level of roughly 50 micrograms.

There is strikingly little difference between the effects of 63, 125 and 1000 micrograms of methylcholanthrene. These curves in Fig 1 seem to have a sigmoid shape.

The mean latency time which in this work is calculated as the mean time between the treatment and the occurrence of each single tumour, varies very little between the three highest dosages but is a little longer for the few papillomas in the groups with the two lower dosages. The average time of occurrence of the first three papillomas however, is much longer for the two lowest dosages than for the three higher doses. The two highest dosages provoked a small number of carcinomas.

c) *Five consecutive applications of methylcholanthrene* The results are presented in Table 3 and in Fig 2. It is seen that only the lowest concentration 1/64 per cent, provoked relatively few papillomas in few of the mice. The five applications of 1/32 per cent solution resulted in a

dilutions from the first solution shortly before the applications. An amount of 0.1 ml was applied on the dorsum of each animal by a pipette. The solution spread evenly out on the skin after application. Table 1 shows the amount of methylcholanthrene administered at the different experiments. Repeated applications were given with three days intervals.

TABLE 1

*Amount of 3-methylcholanthrene Administered to each Animal at the Different Experiments*

| Per cent solution | Single application $\mu\text{g}$ | Five applications $\mu\text{g}$ |
|-------------------|----------------------------------|---------------------------------|
| 1/1               | 1000                             | —                               |
| 1/2               | —                                | 2500                            |
| 1/8               | 125                              | 625                             |
| 1/16              | 63                               | 315                             |
| 1/32              | 32                               | 160                             |
| 1/64              | 16                               | 80                              |

For each type of experiment a group of 30 animals was used: 15 males and 15 females. (No significant differences between the responses of the two sexes were observed. All results are therefore given for both sexes in common.)

The animals were examined every 10th day during the observation period of 18 months (78 weeks). Each tumour was recorded and registered as tumour when present for more than 10 days. The animals were kept until death or killed after 18 months observation. Whenever possible (i.e. except for advanced autolysis) an autopsy was performed and the tumours were controlled histologically.

To secure the non carcinogenicity of the solvent a control group of 30 mice was given 20 applications of benzene with ten days intervals and observed as described above.

As conclusions are only drawn from differences that are quite evident, no formal statistical calculations of significance are performed.

## RESULTS

a) *Benzene applications*: No papillomas appeared in this group. One of the animals developed an ulcer on the dorsum after 10 months. This was the only gross evidence of a possible toxicity of benzene to the skin. The ulcer healed up after some weeks, and the animal lived for 2 months more without developing any tumour.

TABLE 2

*Papillomas Appearing on Hairless Mice Skin up to 18 Months after a Single Application of Different Doses of 3-methylcholanthrene in Benzene Solution*

| Methylcholanthrene concentration in per cent | Total number of tumours per number of mice alive at appearance of first tumour | Per cent of mice bearing |            | Average time of occurrence of three first papillomas (in months) | Mean latency time (in months) |
|--|--|--------------------------|------------|--|-------------------------------|
|  |  | Papillomas               | Carcinomas |  |                               |
| 1/64   | 3/29   | 7                        | 0          | 12.5   | 12.5                          |
| 1/32   | 5/29   | 10                       | 0          | 10.0   | 10.2                          |
| 1/16   | 55/30  | 67                       | 0          | 5.0  | 9.4                           |
| 1/8  | 43/30  | 57                       | 3          | 3.7  | 8.4                           |
| 1/1  | 79/30  | 77                       | 3          | 3.5  | 8.7                           |

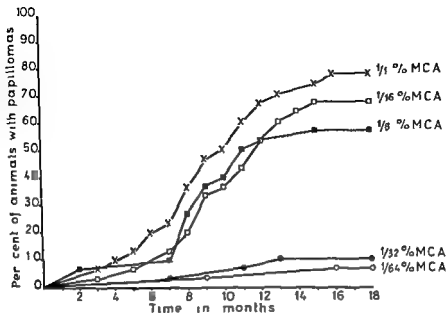


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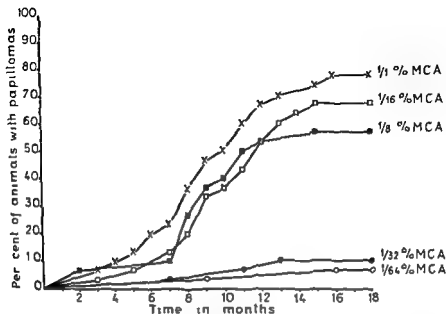


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To secure the non carcinogenicity of the solvent a control group of 30 mice was given 20 applications of benzene with ten days intervals and observed as described above.

As conclusions are only drawn from differences that are quite evident, no formal statistical calculations of significance are performed.

## RESULTS

a) *Benzene applications* No papillomas appeared in this group. One of the animals developed an ulcer on the dorsum after 10 months. This was the only gross evidence of a possible toxicity of benzene to the skin. The ulcer healed up after some weeks, and the animal lived for 2 months more without developing any tumour.

TABLE 2  
*Papillomas Appearing on Hairless Mice Skin up to 18 Months after a Single Application of Different Doses of 3 methylcholanthrene in Benzene Solution*

| Methylcholanthrene concentration in per cent | Total number of tumours per number of mice alive at appearance of first tumour | Per cent of mice bearing |            | Average time of occurrence of three first papillomas (in months) | Mean latency time (in months) |
|--|--|--------------------------|------------|--|-------------------------------|
|  |  | Papillomas               | Carcinomas |  |                               |
| 1/64   | 3/29   | 7                        | 0          | 12.5   | 12.5                          |
| 1/32   | 5/29   | 10                       | 0          | 10.0   | 10.2                          |
| 1/16   | 55/30  | 67                       | 0          | 5.0  | 9.4                           |
| 1/8  | 43/30  | 57                       | 3          | 3.7  | 8.4                           |
| 1/1  | 79/30  | 77                       | 3          | 3.5  | 8.7                           |

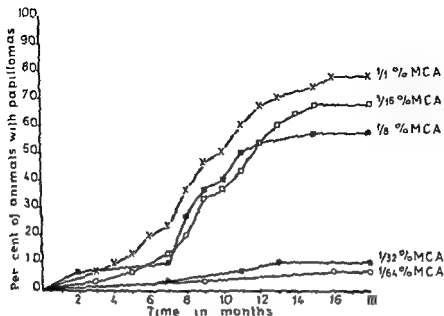


Fig. 1

Percentage of tumor-bearing animals during the observation period after one single painting with methylcholanthrene in benzene at different concentrations

b) *Single application of methylcholanthrene* The results are presented in Table 2 and in Fig. 1. It is seen that whereas the two lowest dosages viz. the 1/64 and the 1/32 per cent provoked very few papillomas in a low percentage of the mice, the three higher dosages produced many papillomas in many of the animals. Evidently, the dose-response curve shows a pronounced increase between the two dosages 1/32 and 1/16 per cent corresponding to a borderline application level of roughly 50 micrograms.

There is strikingly little difference between the effects of 63, 125 and 1000 micrograms of methylcholanthrene. These curves in Fig. 1 seem to have a sigmoid shape.

The mean latency time which in this work is calculated as the mean time between the treatment and the appearance of the first tumor, is longer, the higher the dosage is.

The mean time for the appearance of the first papilloma, however, is much longer for the two lowest dosages than for the three higher doses. The two highest dosages provoked a small number of carcinomas.

c) *Five consecutive applications of methylcholanthrene* The results are presented in Table 3 and in Fig. 2. It is seen that only the lowest concentration 1/64 per cent, provoked relatively few papillomas in few of the mice. The five applications of 1/32 per cent solution resulted in a



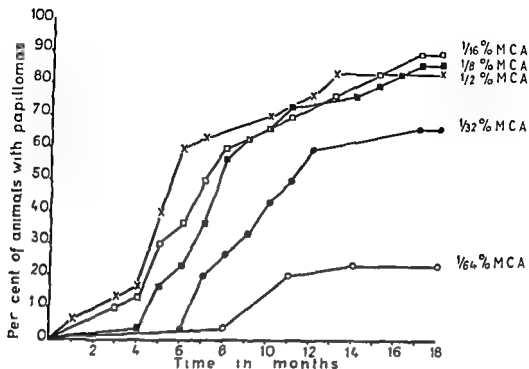


Fig. 2

Percentage of tumour-bearing animals during the observation period after five repeated paintings with methylcholanthrene in benzene at different concentrations

TABLE 3

*Papillomas Appearing on Hairless Mice Skin up to 18 Months after Five Consecutive Applications of Different Doses of 3 methylcholanthrene in Benzene Solution Three Days Intervals between the Applications*

| Methylcholanthrene concentration in per cent | Total number of tumours per number of mice after appearance of first tumour | Percent of mice bearing |            | Average time of occurrence of three first papillomas (in months) | Mean latency time (in months) |
|--|---|-------------------------|------------|--|-------------------------------|
|  |   | Papillomas              | Carcinomas |  |                               |
| 1/64   | 16/21   | 17                      | 0          | 9.7  | 10.6                          |
| 1/32   | 41/21   | 67                      | 0          | 6.0  | 8.0                           |
| 1/16   | 90/30   | 90                      | 1          | 3.5  | 8.0                           |
| 1/8  | 111/25  | 88                      | 8          | 4.7  | 8.7                           |
| 1/2  | 140/17  | 82                      | 10         | 3.0  | 6.3                           |

curve (Fig. 2) very similar to that obtained after a single application of 1/16 or of 1/8 per cent (Fig. 1)

The latency time is a little longer for the lowest dosage than for the others. There is, however, no significant differences between the latency time after a single and after five applications. The average time of occurrence of the three first tumours is also here shorter for the higher dose levels, but there is no marked step between any of the dosages. There is remarkably little difference between the average times of occurrence of the three first papillomas after a single and after five

applications. The three highest dosages provoked some carcinomas the yield coming up to 30 per cent after  $\frac{1}{2}$  per cent solution.

In this group with five applications the mortality of the animals is generally somewhat increased.

## DISCUSSION

When a carcinogenic hydrocarbon dissolved in benzene is applied to the surface of mouse skin a certain amount of it penetrates into the epidermis and some of it further into the corium where a part is resorbed into the general circulation and finally excreted. Many authors have demonstrated that *carcinogens remain in the skin for some time after application*. This carcinogen is eliminated partly through metabolism in the epidermal cells themselves partly through continuous diffusion into the corium and partly through the loss of carcinogen loaded cells from the surface (a more detailed discussion of the fate of the carcinogens after topical application is given by Vorden 1963 and by Iversen & Evensen 1962). An average estimate of the presence of carcinogens in the epidermis after a single application is 7 to 9 days (Engelbreth Holm & S. Iversen 1961). Poel (1969) studied the fluorescence after a single drop of carcinogen it remained for 1 to 3 weeks.

Probably there is a positive correlation between the dose applied and the time of persistence in the skin. Vorden (1963) concluded after his experiments with 3,4-benzpyrene that the length of time during which benzpyrene or its metabolite is present in the epithelial cells is correlated to the amount of benzpyrene applied and to the amount of lipids in the depots (surface, hair canals and sebaceous glands).

Also other factors seem to influence the time of persistence of carcinogens in the epidermis. In the haired mice the hair cycle is of great importance. It has been shown by Berenblum, Haran Ghera & Trainin (1958) that the carcinogens remain much longer in the skin when applied during the resting phase than during the growth phase. This gives a possible explanation of the "hair cycle effect" extensively discussed by Engelbreth Holm and his school (see Borum 1960). A moderate single dose of a carcinogen gives rise to many tumours when applied during the resting phase whereas very few or no tumours are seen when the carcinogen is applied during the growth phase. With very small doses however no difference in the results can be observed and with massive doses the difference is also masked. Andreassen & Borum (1963) have demonstrated that also the grade of the early morphological signs of cellular destruction which follows a single application of a moderate dose of DMBA is dependent on the phase of the hair cycle at the time of application. This may be interpreted in the same way as that also the early cell destruction is dependent upon the time of persistence of the carcinogens in the epidermis.

As regards the hairless mice used in our experiments the hair cycle effect is probably of no or minor importance. The single exposures used

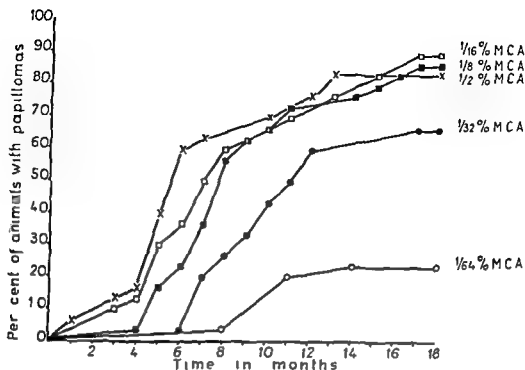


Fig. 2

Percentage of tumourbearing animals during the observation period after five repeated paintings with methylcholanthrene in benzene at different concentrations

TABLE 3

*Papillomas Appearing on Hairless Mice Skin up to 18 Months after Five Consecutive Applications of Different Doses of 3 methylcholanthrene in Benzene Solution 2 three Days Intervals between the Applications*

| Methylcholanthrene concentration in per cent | Total number of tumours per number of mice alive at appearance of first tumour | Per cent of mice bearing |              | Average time of occurrence of three first papillomas (in months) | Mean latency time (in months) |
|--|--|--------------------------|--------------|--|-------------------------------|
|  |  | Papillomas               | 4 carcinomas |  |                               |
| 1/64   | 16/21  | 17                       | 0            | 9.7  | 10.6                          |
| 1/32   | 41/21  | 67                       | 0            | 6.0  | 8.0                           |
| 1/16   | 90/30  | 90                       | 3            | 3.5  | 8.0                           |
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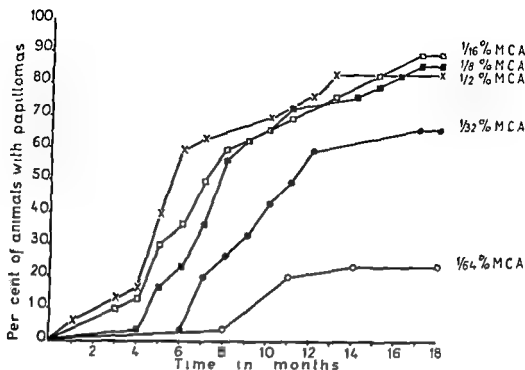


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|--|--|--------------------------|------------|--|-------------------------------|
|  |  | Papillomas               | carcinomas |  |                               |
| 1/64   | 16/21  | 17                       | 0          | 9.7  | 10.6                          |
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| 1/16   | 90/30  | 90                       | 3          | 3.3  | 8.0                           |
| 1/8  | 111/25   | 88                       | 8          | 4.7  | 8.7                           |
| 1/2  | 140/17   | 82                       | 30         | 3.0  | 6.3                           |

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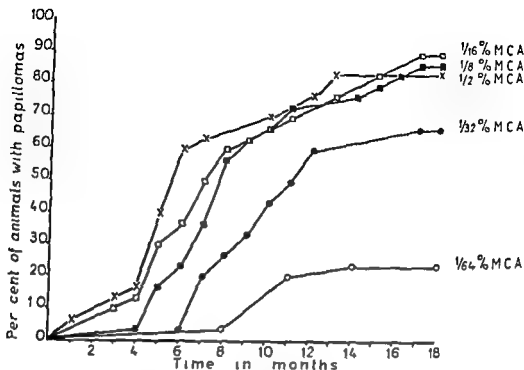


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| 1/16   | 90/30  | 90                       | 3          | 3.5  | 8.0                           |
| 1/8  | 111/25   | 88                       | 8          | 4.7  | 8.7                           |
| 1/2  | 140/17   | 92                       | 30         | 3.0  | 6.3                           |

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in our experiments really meant that the epidermal cells were under the continuous, though probably decreasing, influence of carcinogens for 1 to 3 weeks. In the experiments with 5 applications with 3 days intervals the exposure was at least two weeks longer.

It is also evident that after a single surface application of a certain amount of a carcinogen, we do not know the exact effective dose, i.e. the number of carcinogenic molecules that reaches the supposed critical sites in the cells. At least with higher doses an unknown amount of carcinogen is left on the surface and seems not to penetrate the cells. It may be assumed that the main part of this surface-carcinogen is eliminated from the animal by the acute cell loss demonstrated by *Iversen & Evensen* (1962) and later by *Skjeggstad* (1964). But it may probably be permissible to assume that the real effective dose is positively correlated to the applied dose up to a certain dose level. Further increase in the dose applied is, however, probably not followed by a proportional rise in the effective dose. Only one group of workers (*McCarter, Szerb & Thompson* 1956) has tried to overcome this by using a method which is supposed to give a well-defined dose, washing off the superfluous of carcinogen after a controlled time of application. But these authors have not tested the effect of a single dose. On the contrary, after using a lot of work to apply an initiating controlled dose of a strong carcinogen, they proceeded by applying repeated, uncontrolled doses of an illdefined, weak carcinogen, the croton oil, thereby destroying any possibility to observe the effects of a supposed short exposure to a carcinogen at controlled dose levels; (See also *Ball & McCarter* 1960).

As regards the lower dose levels used in our experiments, we may assume a proportionality between the applied dose and the effective dose. For the higher dose levels this proportionality is probably lacking. This seems to be the most reasonable explanation of the fact that there is very little difference between the effects of the three highest doses given in our experiments.

With these reservations and this definition of the meaning of a single application, we may conclude that the present study (1) confirms the findings of *Terracini, Shubik & Della Porta* (1960) of the critical dose level at which the carcinogens become fully effective after a single application. Such a critical dose level is in our experiments observed for methylcholanthrene in benzene solution applied on hairless mice. The critical dose level with the technique used is found to be around 50  $\mu\text{g}$ . It is manifested as a substantial increase in the total numbers of papillomas occurring in the percentage of animals with tumours and in a possible difference in the average time of occurrence of the three first papillomas in each animal group. One may ask why earlier investigations have not revealed this fact. A critical examination of the reports, however, makes it clear that the work of *Terracini, Shubik & Della Porta* is the first study of the dose-response curve after a single

exposure to carcinogens which takes the hair cycle into consideration, which uses enough animals in each experimental group, and which uses a long enough observation time

The second problem we set out to study was if the possible critical dose level as regards the tumour yield was located at the same dose niveau as the "step" in the tetrazolium test (ii) This was found to be the fact The critical dose level is in both investigations located between the concentrations of 1/32 and 1/16 per cent methylcholanthrene in benzene *Evensen* (1962) has also observed a similar "step" in the effect of a single application of different doses of methylcholanthrene on the rate of cell proliferation in the epidermis *Iversen & Evensen* (1962) have interpreted the changes they observe as being signs of cellular destruction, followed by regenerative processes (see also *Iversen & Bjercknes* 1963)

As in all other studies using small single applications of a carcinogen, most of the tumours were benign papillomas Some of the tumours regressed, many remained unchanged or with only slow local growth during the observation period, and only a few developed to carcinomas It is, however, generally held that there is a positive relationship between the development of benign papillomas and the eventual development of carcinomas (see, for instance, *Roe* 1956) Such a relationship can also be seen in this investigation (Tables 2 and 3) Even if this is correct it is of course impossible to conclude from the results of the present experiment of single application that a real threshold dose exists in carcinogenesis in other words that there should be some small doses of carcinogens being "non dangerous"

If the results described by *Borum* (1954) is compared to the report by *Andreasen & Borum* (1959) it seems to be a proportionality between the grade of cellular destruction as observed histologically, and the development of papillomas and carcinomas In our experiments there was an accordance between the "step" in the tetrazolium test and the critical dose level as concerns the production of papillomas After all, it may thus be permissible to assume a relationship between the grade of cell destruction provoked by graded, relatively small doses of a

As can be explained in this way, we may conclude that the results of the present investigation give a strong support to the usefulness of the tetrazolium method for testing carcinogenic activity The only presupposition is that the carcinogen must be applied in a dose that is over the "critical dose level", but below an ulcer producing dose

When comparing the results of the experiments with five repeated doses (iii) with those obtained after a single application, the most interesting feature is the change of results for the 1/32 per cent solution After a single administration the papilloma development is negligible, whereas five applications produce a significant number of papillomas

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in 67 per cent of the animals. This difference may depend on the longer persistence of the carcinogen after five applications. But it may as well depend on the higher effective dose level that might be reached after five applications. It would obviously have been of interest to study the effects of a few repeated applications of small doses delivered with much longer intervals. In that way the probable accumulation of carcinogen with subsequent increase in the effective dose level would be avoided, and a relatively stable, long-time effect of a small effective dose could be attained.

A possible, simple, but of course more or less speculative explanation of the critical dose level may be the following. To provoke the changes in the skin that lead to tumour formation, it is necessary that a certain amount of carcinogenic molecules attack a certain amount of critical sites in the cells. These cells are supposed to be the basal cells of the epidermis and its derivatives, or some cells in the corium. To reach these cells the carcinogen must diffuse through the horny layer and through the differentiating cells. When a certain dose level is applied, enough carcinogen reaches the critical sites and persists in the skin for a sufficiently long time. Further increase in the concentration of the solution applied has only slight additional effect because the effective dose is not proportional to the applied dose at higher dose levels. With even further increase in the concentration of carcinogen, the direct toxic effect will dominate, and ulcers develop. It is well known that papillomas and carcinomas usually develop in the rand zone of such ulcers, where the dose might have been sufficiently high, but non-ulcerative.

This way of reasoning refers only to a single painting. The results of the five paintings, as well as the massive evidence from the literature, clearly demonstrate that by repeating the applications it is with the strong carcinogens possible to increase the tumour yield up to nearly 100 per cent.

Such a simple explanation of the critical dose level after a single application may get some support also by the results obtained after five applications. It is seen that the effect of repeating the dose is more pronounced by the lower concentrations, and is small at the higher dose levels.

#### SUMMARY

Groups of 30 hairless mice were given one single application of methylcholanthrene at different dose levels. Other groups of mice were given five repeated applications of the same dose levels of methylcholanthrene.

The development of papillomas and carcinomas in the different groups was observed.

A single application of 1/64 and 1/32 per cent solution of methylcholanthrene provoked a negligible amount of tumours, whereas 1/16

1/8 and 1/1 per cent provoked many tumours in many of the animals. A critical dose level seems to exist.

Five repeated applications resulted in a higher tumour yield in all groups. Only the 1/64 per cent solution provoked relatively few tumours.

The significance of these findings are discussed and related to previous reports concerning the tetrazolium test for carcinogenicity.

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# MORPHOLOGICAL CHANGES IN BOVINE AND HUMAN FIBROBLASTS EXPOSED TO TWO STRAINS OF ROUS SARCOMA VIRUS IN VITRO<sup>1</sup> \* 2

By

BJÖRN STENKVIST and JAN POSTÉN

Received 10 iv 64

In recent years the number of species susceptible to Rous sarcoma virus *in vivo* has been found to include not only fowl but also a variety of mammals (1-13) including monkeys (14). Marked differences between various strains of RSV have been established. Ahlstrom *et al* comparing RSV<sub>Mitt Hill</sub> with RSV<sub>Schmidt Rupp</sub> found that the former strain induced sarcoma only in chickens whereas the latter gave rise to tumours also in rats, mice, rabbits, hamsters and guinea pigs (1, 2, 4, 13).

Comparatively little is known about the *in vitro* host range of various strains of RSV with the exception of RSV<sub>Schmidt Rupp</sub> which has been reported to produce round cell foci in rat, mouse and guinea pig cells (4, 15). Recently Svoboda & Chyle have described 'malignization' of rat embryonic cells exposed to strain Prague of RSV (16).

The results of exposing bovine and human fibroblasts *in vitro* to two different strains of RSV are given in this report.

## MATERIALS AND METHODS

### Virus Strains

RSV (SR). For this strain of Rous sarcoma virus we are indebted to Dr C G Ahlstrom Lund Sweden who provided us with a sample in 1963. The derivation history and main biological characteristics of this strain are given in references 1, 2, 4, 6 and 13.

In our laboratory RSV (SR) has been kept in passage in young White Leghorns.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

<sup>1</sup> The following abbreviations have been used: RSV - Rous sarcoma virus; SR - Schmidt Rupp; Su - Su.



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Figs 1 2

- Fig 1** Uninfected control culture of the bovine lung fibroblast strain B1. Note the regular growth pattern characteristic of normal fibroblasts. Unstained culture.  $\times 50$ .
- Fig 2** Control cells from an unexposed culture of calf fibroblasts. Note regular arrangement of elongated cells with monomorphic oval nuclei with several nucleoli. Bovine fibroblasts differ from human fibroblasts by the presence of 6-10 rather than 2-4 nucleoli. Coverslip culture. May-Greenwald-Giemsa.  $\times 670$ .

**RSV (IH)** This strain of Rous sarcoma virus was obtained from Engelbreth Holm in 1953. Its history prior to 1953 is not accurately known. It is probable that the virus is derived from the original Rous (chicken tumor No. 1 strain (17) and was kept in Engelbreth Holm's laboratory for about a decade prior to 1953.

The strain gives rise to tumours in chickens morphologically indistinguishable from those caused by the high titre RSV strain developed by Bryan (18). After rapid passage through about 20 generations of chicks the yield of RSV (IH) from the tumours is high ( $TD_{50} \approx 10^6$ ). Undiluted virus will cause palpable tumours after 5 days (Ponten unpublished).

The RSV (IH) strain was to a limited extent tested *in vivo*.

40 newborn mice of the strain NMRI were injected with minced crude chicken tumour tissue and were observed for 3 months.

18 newborn non inbred rats were also injected with a similar tissue and observed for 3 months.

None of the animals developed tumours or other lesions during the observation period. When autopsied at the end of the observation period no pathological lesions were found.

These experimental data are limited but compatible with RSV (IH) belonging to the category of RSV strains with a limited host range *in vivo*.

### *Preparation of Virus Pools*

Tumours were collected 4-5 days after they had become palpable. The tumour tissue was suspended in PBS and homogenized at 23 000 rpm in a VirTis homogenizer in an ice bath for 2 minutes. The suspension was then centrifuged  $2 \times 20$  minutes at 5 000 rpm. Finally the supernatant was filtered through a millipore filter of 0.45  $\mu$  pore size. The pools were stored in ampoules at  $-65^\circ\text{C}$ .

The biological activity of the RSV (IH) and the RSV (SR) pools was tested by wing web inoculation of samples into week old chicks. With both preparations 10-10 birds developed sarcomas within 9 days.

### *Tissue Culture Methods*

**Medium** Eagle's medium (19) with 10 per cent calf serum.

**Subcultivation** The cell strains whether infected by virus or not were kept in medium and subcultured as soon as the medium was changed, i.e. 1-4 days after each passage.

**Exposure to virus** After removal of medium the monolayers were exposed to 0.3 ml of undiluted virus for 1 hour at  $37^\circ\text{C}$ . Control cultures were sometimes exposed to PBS but this procedure was omitted later when no differences were demonstrated between control bottles left untreated or exposed to 0.3 ml of PBS.

**Incubation** All cultures were incubated at  $37^\circ\text{C}$ . Coverslip preparations (see below) were incubated in 5 per cent CO<sub>2</sub> in humidified air at  $37^\circ\text{C}$ .

**Morphological observation** At each subcultivation a coverslip culture was prepared in a small Petri dish. Coverslips were fixed in methanol and stained routinely according to May-Greenwald-Giemsa. Some cultures were also stained with the Feulgen method.

### *Cell Strains*

Two human (H1 and H2) and two bovine (B1 and B2) strains of fibroblast like cells derived from trypsinized lung tissue were used.

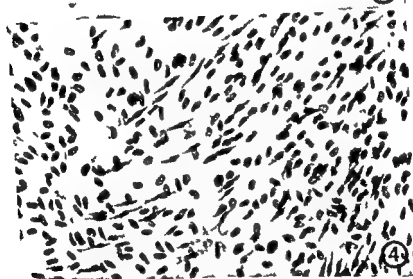
H1 and H2 were developed from a 5 months and 4 months old female foetus respectively. Both were normal and obtained by therapeutic abortion. B1 was developed from a newborn calf and B2 from a calf foetus of an estimated age of 2 months.

## EXPERIMENTAL AND RESULTS

**Control cultures** From each of the four strains (H1, H2, B1 and B2) five sublines were carried independently. All twenty sublines have been subcultured without difficulty and are at present in passage 20-25.



③



④

Figs 3-4

*Fig 3* Basophilic spindle cells from strain H2 of human lung fibroblasts 8 weeks after exposure to RSV (SR). Note very large irregular nucleoli. Coverslip culture. May Greenwald G. emsa.  $\times 1600$ .

*Fig 4* Human lung fibroblasts of strain H2 3 weeks after exposure to RSV (SR). See also typical basophilic spindle cell. Near the upper left corner a small group of round hyperchromatic cells. Note that the altered cells go on top of the normal lightly stained fibroblasts. Coverslip culture. May Greenwald G. emsa.  $\times 160$ .

Their apparent rate of growth has not changed and they have maintained the same morphology. No signs of any alterations or "transformations" have been observed either in the unstained culture vessels or in the stained coverslip preparations removed at each subculture. Spot checks of metaphases have revealed no departure from normal diploidy.

Bovine and human cells derived from trypsinized lung were elongated with pointed ends and had a well-defined homogenous, lightly basophilic cytoplasm and round or oval nuclei of regular form. Variations in the appearance of the individual cells and nuclei were slight. Human cells contained 2-4 small nucleoli in the majority of the cases, whereas calf cells contained about 6-10 nucleoli. Another difference was that the human cells tended to be more elongated than the bovine cells. The cells grew in regular, slightly curved strands in a monolayer. The appearance of the cultures corresponded to the classical description of fibroblast-like cells *in vitro* (Figs 1 and 2).

### *Cultures Exposed to RSV*

One subline of H1, three sublines of H2 and three sublines of B1 were exposed to RSV(SR). One subline of H2, one subline of B1 and one subline of B2 were exposed to RSV(EH).

The ten sublines exposed to virus were carried in parallel with the twenty sublines of control cells using the same medium and the same general handling and tissue culture conditions. Sublines of the same strain receiving the same treatment did not differ significantly from each other.

*Morphological observations.* The alterations in the virus-exposed cultures were complex. To facilitate a description and make comparisons possible, cells were grouped into a few well defined categories. Three types of pathological cells were seen with only a few intermediate forms. The morphology of the three types was similar to that described by Doljanski and Tenenbaum (21, 22) inavian Rous sarcoma cells in tissue culture.

*Basophilic spindle cells.* These cells were slightly smaller than normal fibroblasts but retained an elongated shape. A conspicuous alteration was an intense cytoplasmic basophilia. The nuclei were rounder than those of normal fibroblasts and contained enlarged nucleoli (Fig 3). The basophilic spindle cells typically grew on top of the normal fibroblasts forming easily detectable foci (Fig 4). Mitosis was rare among basophilic spindle cells.

*Basophilic round cells.* These elements had a sparse, well defined and intensely basophilic cytoplasm. Pedunculated cytoplasmic pseudopodia were sometimes observed. The nucleus was often eccentrically placed with a coarse chromatin network and prominent nucleoli (Fig 5 and 6). The diameter of the round cell was slightly larger than the



Figs 3-4

Fig 5 Basophilic spindle cells from strain H2 of human lung fibroblasts 6 weeks after exposure to RSV (SR) Note very large irregular nucleoli Coverslip culture May Greenwald Giemsa  $\times 1600$

Fig 4

Giemsa  $\times 160$

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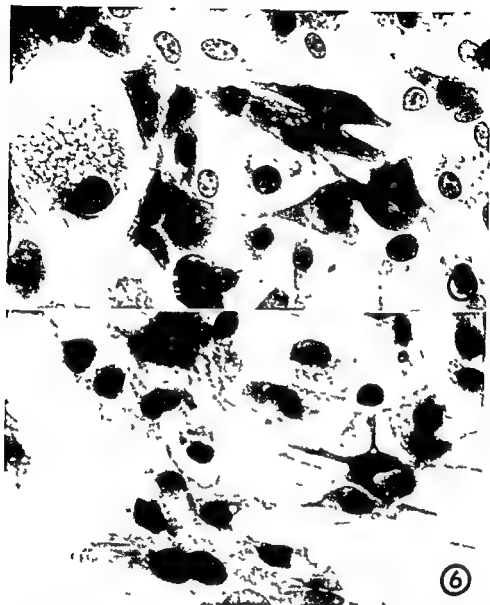
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Figs 3 &amp; 4

*Fig 3* Basophilic spindle cells from strain H2 of human lung fibroblasts 6 weeks after exposure to RSV (SR). Note very large irregular nucleoli. Coverslip culture. May-Grunwald-Giemsa  $\times 1600$ .

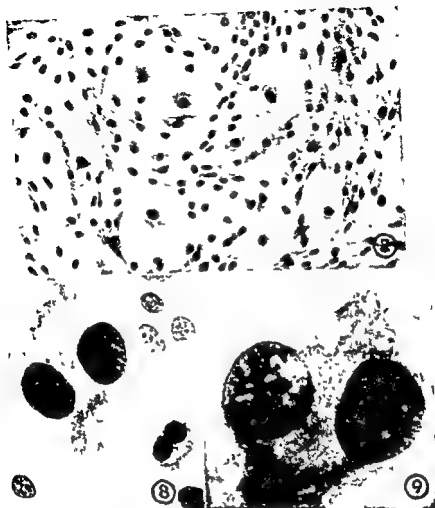
*Fig 4* Human lung fibroblasts of strain H2 3 weeks after exposure to RSV (SR).



Figs 5-6

**Fig 5** Altered cells of different types from strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR). To the *left* a vacuolated giant cell. Most of the cells are basophilic round cells of typical appearance with sparse dark cytoplasm and round often eccentrically placed nuclei with large nucleoli. A group of three parallel basophilic spindle cells can be seen near the *centre* of the picture. Coverslip culture. May Greenwald Giemsa  $\times 620$ .

**Fig 6** Human lung fibroblasts of strain H2 4 weeks after exposure to RSV (SR). High magnification of a focus of altered cells. Note the general similarity to the altered calf cells of Fig 5. The field is dominated by basophilic round cells. Near the *bottom* a binucleated giant cell showing a mirror image type of symmetry. Most nuclei show clearly enlarged nucleoli. Coverslip culture. May Greenwald Giemsa  $\times 620$ .



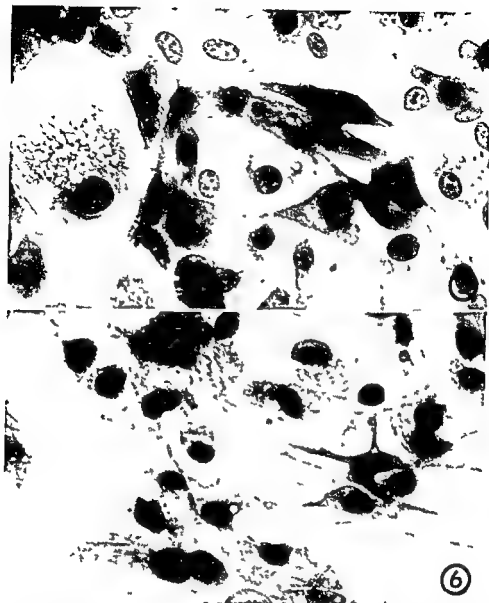
Figs 7-9

*Fig 7* Calf fibroblasts 9 weeks after exposure to RSV (EH). The only recognizable change was the occurrence of scattered vacuolated giant cells. Due to a fixation artefact they have shrunk from the surrounding normal fibroblasts.

*Fig 8*

$\times 670$

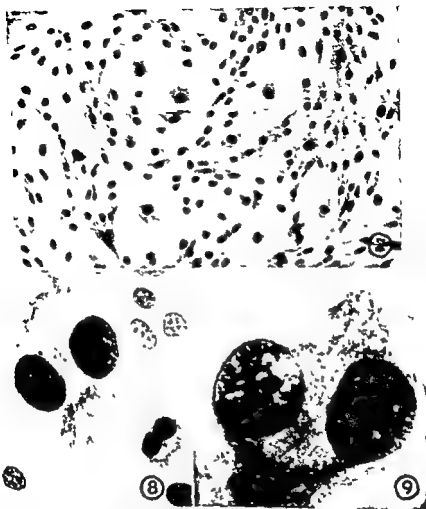
*Fig 9* Same culture as in Fig 8. High magnification of a binucleated "mirror image" cell. Coverslip culture  $\times 1600$ .



Figs 5-6

**Fig 5** Altered cells of different types from strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR). To the left a vacuolated giant cell. Most of the cells are basophilic round cells of typical appearance with sparse dark cytoplasm and round, often eccentrically placed nuclei with large nucleoli. A group of three parallel basophilic spindle cells can be seen near the centre of the picture. Coverslip culture. May Greenwald Giemsa  $\times 620$ .

**Fig 6** Human lung fibroblasts of strain H2 4 weeks after exposure to RSV (SR). High magnification of a focus of altered cells. Note the general similarity to the altered calf cells of Fig 5. The field is dominated by basophilic round cells. Near the bottom a binucleated giant cell showing a mirror image type of symmetry. Most nuclei show clearly enlarged nucleoli. Coverslip culture. May Greenwald Giemsa  $\times 620$ .



Figs 7-9

Fig 7 Calf fibroblasts 9 weeks after exposure to RSV (EH). The only recognizable change was the occurrence of scattered vacuolated giant cells. Due to a

1 culture was Grethwald Giemsa  $\times 160$

Fig 8 Calf fibroblasts of strain B1 III weeks after exposure to RSV (SR). Two binucleated "mirror image" cells of different size are seen. Coverslip culture  $\times 670$

Fig 9 Same culture as in Fig 8. High magnification of a binucleated "mirror image" cell. Coverslip culture  $\times 1600$

TABLE 1

*Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain BI Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Enqelbreth Holm (RSV (FH))*

| Virus   | Cell type                | Time in weeks after exposure to virus |   |   |   |   |   |   |      |       |  |
|---------|--------------------------|---------------------------------------|---|---|---|---|---|---|------|-------|--|
|         |                          | 1                                     | 2 | 3 | 4 | 5 | 6 | 7 | 8-10 | 11-12 |  |
| RSV(SR) | Basophilic spindle cells | —                                     | — | + | + | + | + | + | +    | +     |  |
|         | Basophilic round cells   | —                                     | — | + | + | + | + | + | +    | +     |  |
|         | Vacuolated giant cells   | —                                     | — | + | + | + | + | + | +    | +     |  |
| RSV(FH) | Basophilic spindle cells | —                                     | — | — | — | — | — | — | —    | —     |  |
|         | Basophilic round cells   | —                                     | — | — | — | — | — | — | —    | —     |  |
|         | Vacuolated giant cells   | —                                     | — | — | + | + | + | + | +    | +     |  |

+ = Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes  
 ++ = Pathological cells in every 3rd-4th field of vision (appr corresponding to 3-5 per cent pathological cells)  
 +++ = Pathological cells almost in every field of vision (appr corresponding to 20 per cent of pathological cells)  
 ++++ = In every field of vision numerous pathological cells (appr corresponding 40 per cent pathological cells)

TABLE 2

*Time Sequence of Morphological Changes in Human Cell Cultures of Strain III Exposed to RSV Schmidt-Ruppin (RSV(SR))*

| Virus    | Cell type                | Time in weeks after exposure to virus |   |   |   |   |   |   |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|---|---|---|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5 | 6 | 7 | 8-10 | 11-12 |  |
| RSV (SR) | Basophilic spindle cells | —                                     | — | — | + | + | + | — | —    | —     |  |
|          | Basophilic round cells   | —                                     | — | — | — | — | + | — | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | — | — | + | — | —    | —     |  |

+ = Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes  
 ++ = Pathological cells in every 3rd-4th field of vision (appr corresponding to 3-5 per cent pathological cells)  
 +++ = Pathological cells almost in every field of vision (appr corresponding to 20 per cent of pathological cells)  
 ++++ = In every field of vision numerous pathological cells (appr corresponding 40 per cent pathological cells)

TABLE 3

Time Sequence of Morphological Changes in Human Cell Cultures of Strain H2 Exposed to RSV Schmidt-Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (EH))

| Virus    | Cell type                | Time in weeks after exposure to virus |   |   |   |    |    |    |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|----|----|----|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5  | 6  | 7  | 8-10 | 11-12 |  |
| RSV (SR) | Basophilic spindle cells | —                                     | — | + | + | ++ | ++ | ++ | +    | +     |  |
|          | Basophilic round cells   | —                                     | — | + | + | +  | —  | —  | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | + | +  | —  | —  | —    | —     |  |
| RSV (IH) | Basophilic spindle cells | —                                     | — | — | — | —  | —  | —  | .    | .     |  |
|          | Basophilic round cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |

\* The H2 cells were exposed to RSV (EH) 5 weeks later than to RSV (SR)

— Scattered pathological cells in culture. The rough examination of whole culture necessary to detect changes  
 + Pathological cells in every 3rd 4th field of vision (approx. corresponding to 1-5 per cent pathological cells)  
 ++ Pathological cells almost in every field of vision (approx. corresponding to 20 per cent of pathological cells)  
 +++ In every field of vision numerous pathological cells (approx. corresponding to 40 per cent pathological cells)

TABLE 4

Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B2 Exposed to RSV Engelbreth Holm (RSV (EH))

| Virus    | Cell type                | Time in weeks after exposure to virus |   |   |   |    |    |    |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|----|----|----|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5  | 6  | 7  | 8-10 | 11-12 |  |
| RSV(PII) | Basophilic spindle cells | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|          | Basophilic round cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | + | ++ | ++ | ++ | ++   | ++    |  |

— Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes  
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TABLE 1

*Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B1 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Engelbreth Holm (RSV (EH))*

| Virus    | Cell type                | Time in weeks after exposure to virus |   |   |   |   |   |   |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|---|---|---|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5 | 6 | 7 | 8-10 | 11-12 |  |
| RSV (SR) | Basophilic spindle cells | —                                     | — | + | + | + | + | + | +    | +     |  |
|          | Basophilic round cells   | —                                     | — | + | + | + | + | + | +    | +     |  |
|          | Vacuolated giant cells   | —                                     | — | + | + | + | + | + | +    | +     |  |
| RSV (EH) | Basophilic spindle cells | —                                     | — | — | — | — | — | — | —    | —     |  |
|          | Basophilic round cells   | —                                     | — | — | — | — | — | — | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | + | + | + | + | +    | +     |  |

+ = Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes.  
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 +++ = Pathological cells almost in every field of vision (appr corresponding to 20 per cent of pathological cells).  
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TABLE 2

*Time Sequence of Morphological Changes in Human Cell Cultures of Strain M1 Exposed to RSV Schmidt Ruppin (RSV (SR))*

| Virus    | (cell type)              | Time in weeks after exposure to virus |   |   |   |   |   |   |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|---|---|---|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5 | 6 | 7 | 8-10 | 11-12 |  |
| RSV (SR) | Basophilic spindle cells | —                                     | — | — | + | + | + | — | —    | —     |  |
|          | Basophilic round cells   | —                                     | — | — | — | — | + | — | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | — | — | + | — | —    | —     |  |

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TABLE 3  
Time Sequence of Morphological Changes in Human Cell Cultures of Strain H2 Exposed to RSV Schmidt Ruppin (RSV (SR)) and RSV Ingelbreth Holm (RSV (FH))

| Virus    | Cell type                | Time in weeks after exposure to virus |   |   |   |    |    |    |      |       |  |
|----------|--------------------------|---------------------------------------|---|---|---|----|----|----|------|-------|--|
|          |                          | 1                                     | 2 | 3 | 4 | 5  | 6  | 7  | 8-10 | 11-12 |  |
| RSV (SR) | Basophilic spindle cells | —                                     | — | + | + | ++ | ++ | ++ | +    | +     |  |
|          | Basophilic round cells   | —                                     | — | + | + | +  | —  | —  | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | + | +  | —  | —  | —    | —     |  |
| RSV (FH) | Basophilic spindle cells | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|          | Basophilic round cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|          | Vacuolated giant cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |

The H2 cells were exposed to RSV (FH) 5 weeks later than to RSV (SR).  
 + — Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes.  
 ++ — Pathological cells in every 3rd-4th field of vision (approx. corresponding to 3-5 per cent pathological cells).  
 +++ — Pathological cells almost in every field of vision (approx. corresponding to 20 per cent of pathological cells).  
 ++++ — In every field of vision numerous pathological cells (approx. corresponding to 40 per cent pathological cells).

TABLE 4  
Time Sequence of Morphological Changes in Bovine Cell Cultures of Strain B2 Exposed to RSV Ingelbreth-Holm (RSV (FH))

| Virus   | Cell type                | Time in weeks after exposure to virus |   |   |   |    |    |    |      |       |  |
|---------|--------------------------|---------------------------------------|---|---|---|----|----|----|------|-------|--|
|         |                          | 1                                     | 2 | 3 | 4 | 5  | 6  | 7  | 8-10 | 11-12 |  |
| RSV(11) | Basophilic spindle cells | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|         | Basophilic round cells   | —                                     | — | — | — | —  | —  | —  | —    | —     |  |
|         | Vacuolated giant cells   | —                                     | — | — | + | ++ | ++ | ++ | ++   | ++    |  |
|         |                          | —                                     | — | — | — | —  | —  | —  | —    | —     |  |

+ — Scattered pathological cells in culture. Thorough examination of whole culture necessary to detect changes.  
 ++ — Pathological cells in every 3rd-4th field of vision (approx. corresponding to 3-5 per cent pathological cells).  
 +++ — Pathological cells almost in every field of vision (approx. corresponding to 20 per cent of pathological cells).  
 ++++ — In every field of vision numerous pathological cells (approx. corresponding to 40 per cent pathological cells).





width of the normal fibroblasts. These cells occurred either in foci or diffusely scattered over the culture. They tended to detach easily. Mitosis was rare.

*Vacuolated giant cells* (Figs 7, 8 and 9). These cells showed great variations in size from approximately the size of a normal fibroblast to monster cells approaching 0.5 mm in diameter. The cytoplasm usually contained numerous vacuoles of different size. In some cells a perinuclear arrangement of the vacuoles was found, in others they were diffusely scattered. The cytoplasm showed variable degree of basophilia, which, however, rarely was as intense as that of the previous two cell types. The nuclei were large and often lobulated and in many cells several large nucleoli were seen. Many bi- and tetranucleated cells showed a symmetric arrangement of the nuclei (Figs 8 and 9).

The sequence of the appearance of pathological cells in bovine and human cultures exposed to RSV(SR) and RSV(EH) is given in Tables 1-4.

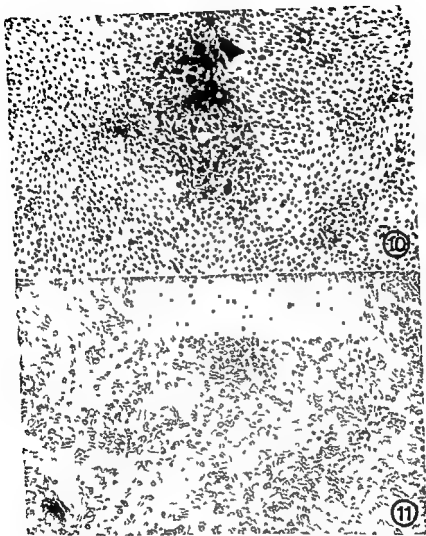
### *RSV Schmidt-Ruppin Series*

Three weeks after exposure, the three sublines of the B1 cell strain of calf fibroblasts exposed to RSV(SR) began to show foci of basophilic spindle and round cells, which increased rapidly in number (Table 1). At the same time a moderate amount of vacuolated giant cells appeared. By 8 weeks the cultures seemed to have reached a stable state characterized by the presence of a mixture of both morphologically normal fibroblasts and basophilic spindle or round cells and vacuolated giant cells. The altered cells tended to occur in foci (Fig 10). Particularly, the basophilic cells tended to grow on top of other cells forming irregular sheets or piles (Figs 11, 12 and 13). In spite of the profound morphological alterations, the over-all growth rate of the cultures did not change materially.

The subline of the H1 cell strain (human lung fibroblasts) exposed to RSV(SR) showed the following changes.

Four to six weeks after infection a small number of foci consisting of a few basophilic spindle cells were observed (Table 2). In the six-week-samples a small number of basophilic round cells and vacuolated giant cells was also seen. In contrast to the cultures of bovine cells, the altered human cells of strain H1 disappeared rapidly so that cover slips 7 weeks or later after exposure to virus were morphologically normal.

The three sublines of the H2 human lung fibroblast strain exposed to RSV(SR) began to show foci of basophilic spindle and round cells three weeks after exposure to virus (Table 3). A small number of such foci were still seen at the time of writing i.e. 12 weeks after exposure. Basophilic round cells and vacuolated giant cells were seen only during a few weeks in the beginning of the phase when the cultures started to change morphologically.



Figs 10 11

- Fig 10** Strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR). Typical focus of altered cells sharply distinguished from surrounding fibroblasts of normal appearance. Coverslip culture. May-Greenwald-Giemsa  $\times 50$ .
- Fig 11** Bovine lung fibroblasts of strain B1 7 weeks after exposure to RSV (SR). Note large numbers of abnormal cells, most of which are highly refractile cells corresponding to the "basophilic round cells" (Table 1). Unstained culture  $\times 80$ .

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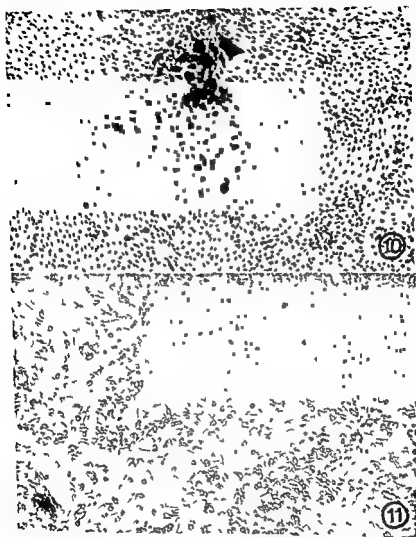
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The three sublines of the H2 human lung fibroblast strain exposed to RSV(SR) began to show foci of basophilic spindle and round cells three weeks after exposure to virus (Table 3). A small number of such foci were still seen at the time of writing i.e. 12 weeks after exposure. Basophilic round cells and vacuolated giant cells were seen only during a few weeks in the beginning of the phase when the cultures started to change morphologically.



Figs 10 11

- Fig 10 Strain B1 of bovine lung fibroblasts 3 weeks after exposure to RSV (SR). Typical focus of altered cells sharply distinguished from surrounding fibroblasts of normal appearance. Coverslip culture. May-Greenwald-Giemsa  $\times 50$ .
- Fig 11 Bovine lung fibroblasts of strain B1 7 weeks after exposure to RSV (SR). Note large numbers of abnormal cells, most of which are highly refractile cells corresponding to the "basophilic round cells" (Table 1). Unstained culture  $\times 50$ .





*Figs 12-13*

- Fig 12* Bovine lung fibroblasts of strain B1 7 weeks after exposure to RSV (SR) High magnification of an accumulation of the round highly refractile cells characteristic of the RSV (SR) effect Unstained culture  $\times 160$
- Fig 13* Calf fibroblasts of strain B1 10 weeks after exposure to RSV (SR) Highly abnormal culture with a mixture of cells characteristic of the balanced state reached about 8 weeks after exposure to virus Normally appearing cells are intermingled with basophilic darkly stained cells of different shapes which tend to accumulate in small heaps Coverslip culture May Greenwald Giemsa  $\times 160$

Both human cell strains showed no difference in their over-all growth rate compared to uninfected parallel control cultures

### *RSV Engelbreth Holm Series*

The two bovine cell strains B1 (Table 1) and B2 (Table 4) exposed to RSV(EH) showed similar responses. Vacuolated giant cells appeared 4 weeks after infection and have persisted for 8 weeks at the time of writing

In contrast to the effects caused by RSV(SR) no basophilic spindle or round cells were observed in the cultures exposed to RSV(EH)

One subline of the human strain H2 exposed to RSV(EH) has not shown any abnormalities during a seven week period of observation at the time of writing

## DISCUSSION

Human and bovine cells exposed to the Schmidt-Ruppin strain of RSV responded with cytological alterations similar to those previously described in RSV infected chicken (21, 22, 26) or murine (15, 16) cells. The changes were complex but three distinct types of pathological elements (basophilic spindle cells, basophilic round cells and vacuolated giant cells) could be discerned. This similarity between changes in avian and murine cells on the one side and bovine and human cells on the other, indicates that the same basic phenomena may be evoked in the four systems by exposure to at least certain strains of RSV.

A wide host range comparable to that of RSV(SR) including cells of such a zoologically distant origin as avian, murine, bovine and primate tissues has not been demonstrated for other tumour viruses. It indicates that the RSV(SR) strain is endowed with a property that would make extensive spread in nature possible.

Cytologically the RSV induced alterations differed in a characteristic way from morphological changes induced by other tumour viruses (27, 28, 29, 30). With polyoma virus in mouse and hamster cell cultures a pronounced "criss-cross" growth pattern seems to be the most characteristic feature (27). SV 40 transformation on the other hand seems to involve a lack of "inhibition of mitosis" and the appearance of epithelial like cells as a primary change (28, 29, 30).

Another difference was the failure of RSV altered cells to 'breed true'. In human cell cultures the proportion of altered cells decreased on prolonged serial cultivation. In the bovine system they remained at an approximately stable level for a long period and were still mixed with cytologically normal fibroblasts even after 12 weeks of observation. The mechanism responsible for the maintenance of this balance is unknown. It may be that altered cells have no select

ed cells may then be counteracted by a slow cytopathogenicity. The fast type of growth mechanism is principally the same as that proposed for Rous sarcoma transplanted by cells in an isologous *in vivo* system (31).

Two differences in the activity of RSV(SR) and RSV(EH) were observed. The latter strain failed to alter human fibroblasts whereas the former evoked changes in the same system. In calf fibroblasts both strains gave rise to alterations. The difference between the two virus strains in their effects on calf fibroblasts was qualitative, since cultures exposed to RSV(SR) displayed the complete spectrum of morphological changes, whereas calf fibroblasts exposed to RSV(EH) only showed vacuolated giant cells but no basophilic spindle or round cells. We do not want to emphasize the importance of the latter difference between the two virus strains, since we have observed a full spectrum of alterations in bovine kidney cultures exposed to RSV(EH) (unpublished).

Another reason to avoid premature conclusions concerning the activity of different RSV preparations is the demonstration by Rubin and coworkers (23, 24) of an intimate functional relationship between such members of the avian tumour virus complex as RSV, RIF and RAV. Crude tumour filtrates were used in the present work and it is not known whether the effects observed represent the action of one or several different viruses belonging to the avian tumour virus complex.

#### SUMMARY

Human and bovine fibroblast-like cells cultured from lung have been exposed to Rous sarcoma virus *in vitro*. The Schmidt-Ruppin and the Englebreth-Holm strains of RSV were used.

With the Schmidt-Ruppin strain human and bovine tissue cultures responded with cellular morphological alterations similar to previously described changes in chicken and murine cells exposed to RSV. Basophilic spindle and round cells as well as vacuolated giant cells appeared between 3-6 weeks after exposure to virus. These abnormal cells appeared in greater proportion in bovine than in human fibroblast cultures. In the bovine cultures the pathological cells reached a stable state with about 40 per cent abnormal cells in the cultures. In the human cultures the pathological cells increased in number initially. With time the proportion of morphologically altered cells decreased and in one strain they seemed to disappear altogether.

The Englebreth-Holm strain of RSV did not evoke any alterations in human cells. In calf cell cultures vacuolated giant cells but no basophilic spindle or round cells were noted.

Control cultures carried under the same conditions *in vitro* as the cultures exposed to RSV displayed no morphological alterations.

It is concluded that the Schmidt-Ruppin strain of RSV is able to alter human and bovine cells in a way cytologically indistinguishable from the manner in which chicken and rat cells have been reported to "transform" after exposure to RSV

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# ANTIBODY RESPONSE TO TYPE 1 ORAL POLIO VACCINE

By

INGER PETERSEN and HEDDIS VON MAGNUS

Received 4 ix 64

In April-May 1963 a vaccination campaign with oral polio vaccine type 1 was carried out in Denmark (1,8). In connection with this program, at study of the antibody formation in 297 individuals was performed. The present paper records the results of this study.

## MATERIALS AND METHODS

**Vaccine.** Monovalent type 1 oral poliovirus vaccine prepared from the Sabin strain LSc 2ab by the Connaught Laboratories Toronto Canada was used. The vaccine was bought concentrated and was diluted in a mixture of equal amounts of 2 molar

to room temperature for 3 hours before actual use.

Each person was given 3 drops of vaccine on a lump of sugar. Young children received the vaccine in 30 per cent syrupus sacchari on a disposable plastic spoon.

**Collection of serum.** Blood samples for antibody titrations were obtained before and 5-7 weeks after the vaccination from 297 persons aged 5 months to 68 years.<sup>1</sup>

**Antibody titrations.** Paired serum samples from all 297 persons were titrated for type 1 polio virus neutralizing antibodies. In addition the sera from 135 of these individuals (selected at random) were titrated for type 2 and 3 antibodies.

The virus strains employed in the neutralization tests were type 1 Brunhilde

the virus serum mixture was inoculated into each of two tissue culture tubes with a confluent monolayer of primary monkey kidney cells and 18 ml of medium 199. The tubes were placed in roller drums or stationary racks (usually roller drums for the first 3 days after seeding and thereafter in stationary racks).

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Some tests also comprised a titration of the WHO International Reference Preparations for anti poliomyelitis serum types 1, 2 and 3. From 22 titrations of each type of serum it was found that the geometric mean titre of the Int. Ref. Serum type 1 was 90, type 2 had a titre of 150 and type 3 a titre of 60.

<sup>1</sup> The sera were kindly supplied by the Statens Serum Institut, Copenhagen, Denmark.  
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in about one fourth of the individuals while the majority showed no change in antibody titre

TABLE 1

*Type 1 Conversion Rate in Various Age Groups after Oral Type 1 Vaccination*

| Age   | No  | Type 1 negative |       |        |       | Conversion rate |
|-------|-----|-----------------|-------|--------|-------|-----------------|
|       |     | Before          | After | Before | After |                 |
| <1    | 12  | 3 (7)*          | 2     |        |       |                 |
| 1     | 27  | 9               | -     |        |       |                 |
| 2     | 9   | 0 (1)†          | 1     | 17/49  | 3/49  | 14/17 = 82 %    |
| 3     | 1   | 0               | -     |        |       |                 |
| 4     | 12  | 3               | -     |        |       |                 |
| 5     | 1   | 1               | -     |        |       |                 |
| 6     | 17  | 8               | -     |        |       |                 |
| 7     | 12  | 4               | -     |        |       |                 |
| 8     | 17  | 7               | -     | 38/100 | 0/100 | 38/38 = 100 %   |
| 9     | 14  | 5               | -     |        |       |                 |
| 10    | 12  | 4               | -     |        |       |                 |
| 11    | 15  | 6               | -     |        |       |                 |
| 12    | 12  | 1               | -     |        |       |                 |
| 13    | 13  | 0               | -     |        |       |                 |
| 14    | 14  | 0               | -     |        |       |                 |
| 15    | 9   | 2               | 1     |        |       |                 |
| 16    | 4   | 0               |       | 4/69   | 2/69  | 2/4 (= 50 %)    |
| 17    | 3   | 0               |       |        |       |                 |
| 18    | 4   | 1               | 1     |        |       |                 |
| 19    | 6   | 0               | -     |        |       |                 |
| 20    | 4   | 0               | -     |        |       |                 |
| 21-30 | 20  | 1               | -     |        |       |                 |
| 31-40 | 18  | 0               | -     | 2/79   | 1/79  | 1/2 (= 50 %)    |
| >40   | 41  | 1               | 1     |        |       |                 |
|       | 297 | 58 (61)         | 6     |        |       |                 |

\* 2 children had a maternal antibody titre of 6 before vaccination (see Table 3)

† This child had an antibody titre of 8 before vaccination and no antibodies after vaccination

In Table 1 the conversion rate from type 1 negative to type 1 positive is shown for the various age groups included in the study. It will be seen that in the age group under 4 years, 14 out of 17 type 1 negative

lost type 1 antibodies after the oral vaccination

In the age group 4-11 years, all 38 children without type 1 antibodies before vaccination responded to the oral vaccine with formation of type 1 neutralizing antibodies, the conversion rate thus being 100 per cent

In the age groups of 12 years and older only 6 out of 148 individuals lacked type 1 antibodies before vaccination. Three of these 6 types 1

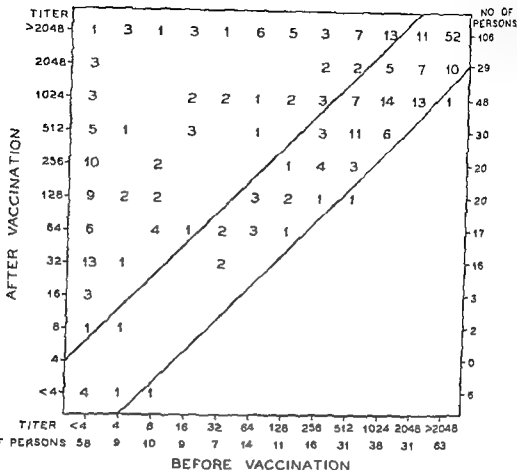


Fig 1

Type 1 antibody response of 297 persons aged 5 months-68 years after feeding of monovalent type 1 Sabin vaccine

### RESULTS

**Type 1 antibody response** Fig 1 shows the type 1 neutralizing antibody titres before and 5-7 weeks after vaccination. It will be seen that the homologous antibody response was very satisfactory. Of 58 individuals without type 1 antibodies before vaccination, only 4 remained negative after vaccination, and the great majority showed antibody titres of 32 or higher, the geometric mean titre being 120.

Of the 28 individuals with antibody titres of 4-16 before vaccination, 25 showed an increase in antibody level with titres ranging from 32 to >2048 after vaccination. The remaining 3 persons, with low antibody levels, however, did not react to the vaccination and two of them (a 6 months old child without previous Salk vaccination and a 2 year-old child who had received the 4th Salk vaccination 1 year before) showed an antibody titre of <4 after vaccination.

When the pre-vaccinal antibody level was higher (i.e. more than 32), the picture was different in that an antibody rise was demonstrated only

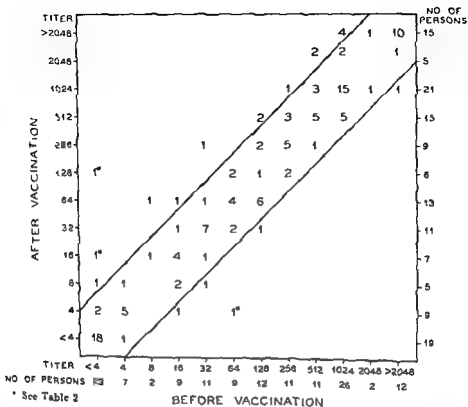


Fig 3

Type 3 antibody response of 135 persons aged 5 months-68 years after feeding of monovalent type 1 Sabin vaccine

Group has been recorded. This figure shows the effect of the oral type 1 vaccination, which is particularly striking in the age group 4-11 years.

In Fig 5 the geometric mean of type 1 antibody titres before and after vaccination has been correlated with the age of the individuals. It will be seen that the geometric mean titre after vaccination ranged from 300 to 1700 for all age groups except for the very youngest under one year, who had a mean antibody titre of 114.

**Heterologous Antibody Response** 135 of the 297 paired sera were selected at random for study of type 2 and type 3 neutralizing antibodies.

Fig 2 shows that a number of individuals had a moderate heterologous type 2 antibody response to the oral type 1 vaccine. In 14 persons the rise in titre was more than 4-fold. A 4 fold change in titre is within the experimental error. Since, however, twelve persons showed a 4 fold rise while only 3 individuals showed a 4-fold decrease in titre, this finding seems to indicate a slight heterologous response also in some of these individuals.

In Fig 3 the type 3 antibody titres are recorded, and it is evident

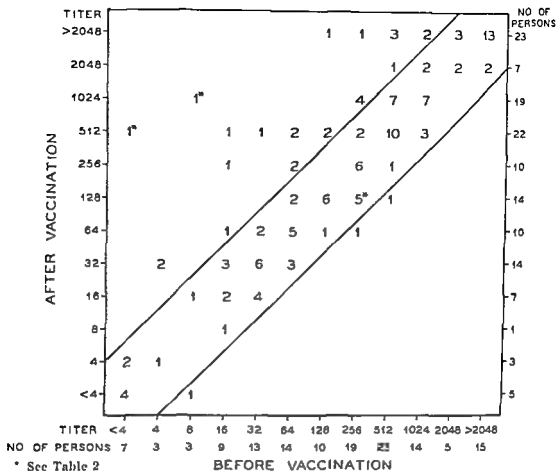


Fig 2

Type 2 antibody response of 135 persons aged 5 months-68 years after feeding of monovalent type 1 Sabin vaccine

negative persons responded to the vaccination, and only  $3/148 = 2$  per cent were without type 1 antibodies after the oral vaccination

In Table 3, the type 1 antibody response in 12 children under one year of age is listed individually together with their Salk vaccination status. Nine of these children responded to the vaccination with good levels of type 1 antibodies, while the result for the remaining 3 children was unsatisfactory. Child No. 8909, 5 months old, did not have antibodies neither before nor after feeding of the type 1 vaccine. A second child, No. 11042, showed a low level of maternal antibodies at the time of vaccination, and the antibody titre dropped from 6 to  $<4$  during the following 5 weeks. A third child, No. 11046, had received 3 Salk vaccinations and had a type 1 antibody titre of 90. This child did not have a booster response after feeding of type 1 vaccine, but showed a titre of 64 five weeks after vaccination.

In Fig. 4 the 297 individuals have been divided into groups according to age, and the percentage of individuals with type 1 antibodies before and after vaccination as well as the number of persons in each age

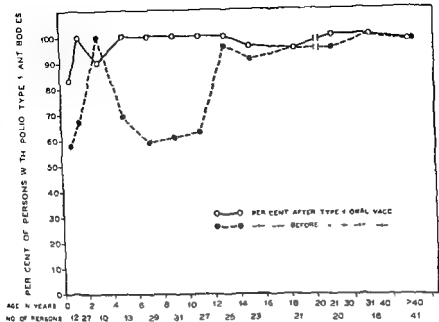


Fig 5

Polio type 1 antibody pattern in various age groups before and after oral type 1 vaccination

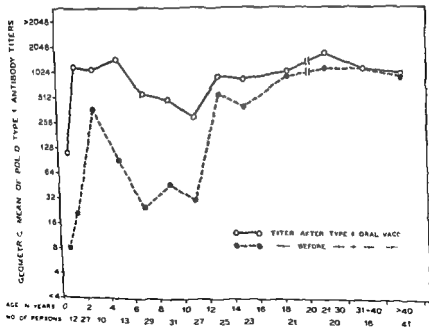


Fig 5

Geometric mean of polio type 1 antibody titers in various age groups before and after oral type 1 vaccination

that the heterologous type 3 antibody response was less pronounced than the type 2 response

Among the 135 persons studied three showed an unusual heterologous antibody reaction. The serum titres for these 3 individuals are in Figs 2 and 3 marked with an asteric and they have also been listed separately in Table 2. The 2 first subjects (Nos 8708 and 8702) both aged 6 years show very pronounced heterologous antibody response, in spite of the fact that 8708 was triple negative before vaccination and 8702 had only type 2 pre-vaccinal antibodies. The third person, No 8969, 10 years old, very unexpectedly show a 16-fold drop in type 3 antibodies in relation to type 1 oral polio vaccination. It seems very unlikely that these irregular findings can be real. Probably a mislabeling of the sera has taken place.

TABLE 2  
3 Children with Unusual Heterologous Antibody Response to Type 1 Oral Polio Vaccination

| No   | Age in years | Antibody titres |        |    |            |        |     |
|------|--------------|-----------------|--------|----|------------|--------|-----|
|      |              | Before vacc     |        |    | After vacc |        |     |
|      |              | 1               | Type 2 | 3  | 1          | Type 2 | 3   |
| 8708 | 6            | 0*              | 0      | 0  | 360        | 720    | 23  |
| 8702 | 6            | 0               | 11     | 0  | 720        | 1450   | 180 |
| 8968 | 10           | 0               | 256    | 64 | 45         | 128    | 4   |

\* < 4 is recorded as 0

TABLE 3  
Polio Virus Type 1 Antibodies before and after Oral Type 1 Polio Vaccination in Children Aged 5-11 Months

| Nr    | Age in months | Previous Salk vacc | Antibody titre against type 1 |                 |
|-------|---------------|--------------------|-------------------------------|-----------------|
|       |               |                    | Before oral vacc              | After oral vacc |
| 11001 | 5             | 0                  | 0                             | 128             |
| 8909  | 5             | 0                  | 0                             | 0               |
| 11044 | 6             | 0                  | 0                             | 180             |
| 8908  | 6             | 0                  | 0                             | 128             |
| 11043 | 6             | 0                  | 64                            | 180             |
| 11042 | 6             | 0                  | 6                             | 0               |
| 11041 | 6             | 0                  | 0                             | 45              |
| 11003 | 7             | 2                  | 90                            | >2048           |
| 8904  | 8             | 0                  | 0                             | 256             |
| 11046 | 10            | 3                  | 90                            | 64              |
| 11002 | 10            | 3                  | 180                           | >2048           |
| 11047 | 11            | 3                  | 32                            | 1450            |

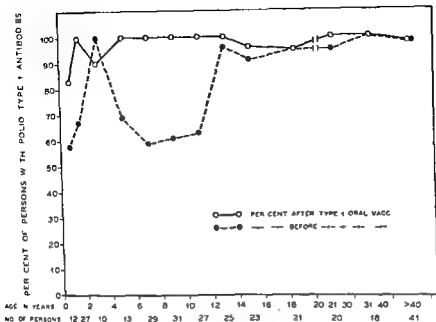


Fig 4

Polio type 1 antibody pattern in various age groups before and after oral type 1 vaccination

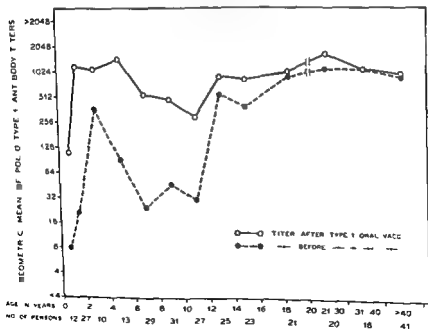


Fig 5

Geometric mean of polio type 1 antibody titers in various age groups before and after oral type 1 vaccination



## DISCUSSION

It is generally accepted that the use of inactivated polio vaccine of the Salk type has had a very pronounced preventive effect on the occurrence of paralytic polio in many parts of the world (3). However, it is known also that the vaccination antibodies decrease significantly already after a few years (4, 5, 6, 7, 11).

Oral polio virus vaccine has been widely used both in unvaccinated and in solidly immunized populations. Both monovalent, bivalent, and trivalent vaccines have been employed and they have all been found to produce antibody formation in a high percentage of the vaccinated individuals provided that a proper virus dose was used (3).

In the present study paired sera from individuals receiving a dose of  $10^{3.3}$  TCID<sub>50</sub> monovalent, oral type 1 polio vaccine (Sabin) were studied in neutralization tests. The material comprised 297 individuals between 5 months and 68 years, the great majority of whom previously had received 2 or 4 vaccinations with inactivated polio vaccine (Salk). It was found that there was a good response to the single dose of oral vaccine. Before the vaccination 58 of the 297 individuals or 20 per cent lacked demonstrable type 1 antibodies while only 6 persons or 2 per cent had no antibodies after vaccination (Table 1). Individuals in the age group 4-11 years who before the vaccination showed the largest percentage of sero-negatives, 38 per cent, reacted particularly well in that all individuals developed type 1 antibodies as a result of the oral vaccination. These findings correspond well with findings in other studies (3, 11, 13).

The antibody levels were also satisfactory. The geometric mean titre of neutralizing antibodies reached 120 in the group of individuals who did not have type 1 antibodies before vaccination. In the neutralization tests the sera were tested against Brunhilde virus. If LSc had been used, the serum titre would have been slightly higher (12).

In accordance with the results obtained by others (9, 10) it was found that the type 1 oral vaccine elicited a moderate heterotypic type 2 antibody response (Fig. 2). A slight heterotypic type 3 response was noted in some individuals (Fig. 3).

## SUMMARY

A dose of  $10^{3.3}$  TCID<sub>50</sub> of Sabin type 1 polio vaccine was given to 297 individuals between the age of 5 months and 68 years. The antibody response was very satisfactory. Of 58 persons (20 per cent) without type 1 antibodies before vaccination, only 6 (2 per cent) remained negative, and the great majority of the type 1 sero-negative individuals reached antibody levels of 32 or higher, the geometric mean titre being 120. The few non responding individuals were partly found in the older age groups and partly in children of 2 months-2 years. In the age group 4-11 years, in which no less than 38 per cent of the individuals



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In the present study paired sera from individuals receiving a dose of  $10^{-3}$  TCID<sub>50</sub> monovalent, oral type 1 polio vaccine (*Sabin*) were studied in neutralization tests. The material comprised 297 individuals between 5 months and 68 years, the great majority of whom previously had received 3 or 4 vaccinations with inactivated polio vaccine (*Salk*). It was found that there was a good response to the single dose of oral vaccine. Before the vaccination 58 of the 297 individuals or 20 per cent lacked demonstrable type 1 antibodies while only 11 persons or 2 per cent had no antibodies after vaccination (Table 1). Individuals in the age group 4-11 years who before the vaccination showed the largest percentage of sero-negatives, 38 per cent, reacted particularly well in that all individuals developed type 1 antibodies as a result of the oral vaccination. These findings correspond well with findings in other studies (3, 11, 13).

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## SUMMARY

A dose of  $10^{-3}$  TCID<sub>50</sub> of *Sabin* type 1 polio vaccine was given to 297 individuals between the age of 5 months and 68 years. The antibody response was very satisfactory. Of 58 persons (20 per cent) without type 1 antibodies before vaccination, only 6 (2 per cent) remained negative, and the great majority of the type 1 sero-negative individuals reached antibody levels of 32 or higher, the geometric mean titre being 120. The few non-responding individuals were partly found in the older age groups and partly in children of 5 months-3 years. In the age group 4-11 years, in which no less than 38 per cent of the individuals

had no type 1 antibodies before the oral vaccination, all the sero negative children showed type 1 antibodies after vaccination

A total of 135 persons were studied for heterotypic antibody formation and it was found that the type 2 response was moderate while the type 3 response was less pronounced

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## BIOLOGICAL ACTIONS OF POLYANIONS

### *A Comparative Study of the Effect on Herpes Virus, Blood Coagulation, Red Blood Cells and Growth Behaviour of Cells on a Glass Surface*

By

ANTTI VAHERI, ILRO IKKALA, ERKKI SAXEN and KARI PENTTINEN

Received 31 III 64

A number of reports have been published on the activities of polyanions in most different biological systems. Various enzymes (Diczfalusy *et al* 1953, Dierick & Stockx 1957, Heymann *et al* 1958 and 1959), viruses (Neher & Krafolder 1955, Penttinen 1956, Stahlmann & Gothofkar 1958, Young & Mora 1960) and tumour growth (Regelson & Holland 1958) have been inhibited. Our present interest in these substances was aroused by the finding that a polyphosphate seemed to exercise an effect on the growth of cells (Saxen & Penttinen 1962) which was very similar to that previously described as due to fresh individual human sera (Penttinen & Saxen 1956 and 1959, Saxen & Penttinen 1961). The cells did not grow in the more common, loose mosaic structures, but in dense clumps only weakly attached to the glass surface. Furthermore, the same polyphosphate has been found to agglutinate red blood cells and to inhibit influenza virus in eggs (Penttinen 1956) and herpes virus in cell culture (Vaheri & Penttinen 1962). Special interest is attached to the observation that heparin, a polyanion of animal origin, seemed to share the biological effect of the above polyphosphate on the growth of cells (Saxen & Penttinen 1962) and on herpes simplex virus (Nahmias & Kibrick 1963, Vaheri & Cantell 1963).

The current comparative studies were embarked upon in an endeavour to determine possible common features in the biological effects of polyanions on herpes simplex virus, blood coagulation, red blood cells and the growth behaviour of cells on a glass surface. To this end, there were available a number of polyanions developed, originally as enzyme inhibitors (Diczfalusy *et al* 1953), in the laboratories of the Leo Company pharmaceutical manufacturers, Helsingborg, Sweden.<sup>1</sup>

The studies, very interesting in principle with respect to the similarity in the effect of fresh human sera and polyanions on cell attachment

Supported by a research grant from the Sigrid Juselius Foundation

<sup>1</sup> We are greatly indebted to Drs B. Hogberg and H. Fex of the Leo Company

ment and growth behaviour on a glass surface, are reported separately (Nordling *et al* 1964 b)

## MATERIAL AND METHODS

### Polyanions

The characteristic properties of the polyanions investigated have been compiled in Table 1<sup>1</sup>

- \* The figures given after the primary and secondary phosphates represent their

†

Leo 114 G)

- ‡ From dextran molecular weight about 60 000

- \* Hydrolysis entails that the primary phosphates in Leo 116 C are columned off. The degree of polymerization is accordingly unchanged

The polyanions 137 B, 161 A, 160 A and 164 A are easily dissolved in water; the other polyanions are soluble in mild alkali.

*Heparin.* A commercial preparation of sodium heparin (Medica Helsinki) containing about 100 IU per mg was used.

*Determination of heparin like antithrombin activity.* A quantity of 0.2 ml citrated bovine plasma (9 parts to 1 part of anticoagulant) and 0.2 ml of the test substance diluted with buffer (Waller's dilution fluid II) were incubated for 3 minutes at room temperature. The mixture was clotted with 0.2 ml of thrombin (Topostasine® La Roche) solution which gave a clotting time of 10–14 seconds in this system with buffer only.

A reference curve was prepared with the aid of known concentrations of heparin (2.5 µg per ml of plasma). For each test substance there were selected two dilutions which gave clotting times fitting the reference curve. From the reference curve the quantity of the test substance was calculated which in this test system had the same

titres of the test substances. op  
ere added to bovine plasma. These  
agent hexadimethrine bromide

(Polybrene®) by Golal's (1961) thrombin system. The results are expressed as quantities of the test substances neutralized by 1 µg of Polybrene®.

at lactalbumin hydrolysate served as  
each dish the final volume was 1 ml  
in a humidified 5 per cent CO<sub>2</sub> atmos  
s the medium was removed and the  
monolayers were supplemented by 2 ml of 15 per cent carboxymethyl cellulose  
overlay medium with no neutral red. After 3–4 days of incubation at 37°C the  
plaques could be  
instances only  
final concentra  
The quantiti

... cent of the number of

<sup>1</sup> Data obtained from the Leo Company

TABLE 1  
*Polymers, Characteristic Anionic and Cationic Groups Approximate Molecular Weight and Hyaluronidase Inhibition*

| Index no | Polymers   | Characteristic anionic and cationic groups* | Molec weight | Hyase in inhibition† |
|----------|--|---|--------------|----------------------|
| 131 A    | Polyvinylalcohol with acid esters of phthalic acid | carboxylates, arom + aliph, ½/unit          | 20000        | 0.1                  |
| 137 B    | Polyphtoroglucinol phosphate                       | phosphates, sec and possibly some prim      | polydisperse | 0.1                  |
| 127 A    | Dextran with acid esters of phthalic acid          | carboxylates, arom, 2/unit                  | 60000g       | 0.2                  |
| 175 A    | Copolymer of styrol and maleic acid, 1:1           | carboxylates aliph                          | 80000        | 0.2                  |
| 116 F    | Polystilbol phosphate of high molec weight         | phosphates prim (10) and sec (70-80)        | > 14000      | 0.6                  |
| 116 C    | Polystilbol phosphate of medium molec weight       | phosphates, prim (25) and sec (60-70)       | 14000        | 0.6                  |
| 153 A    | Formaldehyde polymers of p oxybenzoic acid         | carboxylates, arom                          | ? > 5000     | 0.7                  |
| 114 C    | Polyestradiol phosphate                            | phosphates, prim (30) and sec (60)          | 25000        | 1.0                  |
| 140 A    | Poly bisphenol A phosphate                         | phosphates, prim (20) and sec (60-70)       | ?            | 1.0                  |
| 139 C    | Polystilbol phosphate hydrolyzed                   | phosphates sec and no prim                  | 14000*       | 1.3                  |
| 155 A    | Formaldehyde polymers of p oxybenzoic acid         |   |              |                      |

|   | phosphorus<br>(70-80) | phosphates prim (70) and sec<br>(?)         | 15000    | 15   |
|---|-----------------------|---|----------|------|
| polyethyl phosphate of low molec weight                 |                       |   |          |      |
| polybutyric phosphate                                   |                       | carboxylates arom, basic<br>groups possibly | ? > 5000 | 2    |
| formaldehyde polymer of anthranilic acid                |                       | carboxylates arom                           | < 5000   | 2    |
| Formaldehyde polymer of salicylic acid                  |                       |   | ?        | 3    |
| Polyacetic phosphate                                    |                       | phosphates prim (?) and sec<br>(?)          |          | 5    |
| Pectinic acid with acid esters of phthalic acid         |                       | carboxylates, arom + aliph<br>2/unit        | > 50000  | 20   |
| Dextran with acid esters of phthalic acid               |                       | carboxylates, arom, 1/unit                  | 500000   | 100  |
| Copolymer of methylmethacrylate and maleic<br>acid, 2:1 |                       | carboxylates aliph                          | > 10000  | 175  |
| Rutin esterified with 5:6 acid phthalic acid<br>groups  |                       | carboxylates, arom + aliph,<br>5-6 units    | 1500     | 100  |
| Dextran with acid esters of phthalic acid               |                       | carboxylates arom, 1/unit                   | 510      | 800  |
| Tetra Na salt of stilbol disphosphate                   |                       | phosphates, prim (100)                      | ?        | 1000 |
| Polybutin phosphate of low molecular weight             |                       | phosphates prim, (70) and sec<br>(?)        | > 15000  | —    |
| Polyphlorotin phosphate with also basic<br>groups       |                       | polyampholytic                              |          | —    |
| 176 A   |                       |   |          |      |
| 181 A   |                       |   |          |      |
| 184 A   |                       |   |          |      |
| 182 A   |                       |   |          |      |
| 128 A   |                       |   |          |      |
| 151 A   |                       |   |          |      |
| 164 A   |                       |   |          |      |
| 160 A   |                       |   |          |      |



plaques from the average control figures was calculated as follows. The average percentage of the reduction of the plaque number was arrived at for each concentration of the polymers, and the 50 per cent reducing value was interpolated linearly from these percentages.

**Effect on chicken red cells** The substances were titrated on plastic plates like haemagglutinating agents, using 0.5 per cent chicken red cells and an incubation time of 2 hours at room temperature. Phosphate buffered saline without  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  served as the diluent for the substances and the red cells, both of which were added in a volume of 0.25 ml. The results in Table 2 are given as the minimal 'haemagglutinating' concentration of the polymers diluted in steps.

**The clumping test** HeLa cells maintained in 30 per cent inactivated human serum pool in Hank's salt solution were used. Trypsinization was effected by Difco 1/250 trypsin, with a calculated final concentration of 0.16 per cent of trypsin in Hank's salt solution. The period of incubation was 30 minutes at  $37^{\circ}\text{C}$ . The inoculum size was approximately 30,000 cells. The test tubes (16  $\times$  120 mm) were washed in mild alkali and sterilized in a heat oven (Vordling *et al* 1964a). The polymers were diluted in the culture medium which consisted of 30 per cent aged pooled serum in Hank's salt solution. The growth behaviour was recorded after 24 hours' stationary incubation, strong clumping being recorded when cells were not attached to the glass, but to each other as freely moving large clumps and clear clumping indicating attachment of the cells to the glass surface as dense clumps. The dilution of polyanion sufficient to cause this degree of clumping is given in Table 2. No clumping was recorded for cells growing in a loose structure and forming mosaic-like sheets.

## RESULTS

The results have been compiled in Table 2 which gives the anti-thrombin effect, the effect of Polybrene® titration, metachromatic activity, the rate of herpes virus inhibition, the haemagglutinating activity on chicken red cells (no agglutinating activity was observed when guinea-pig or human red cells were used), and the clumping effect of the polyanions on HeLa cells grown on a glass surface. From these findings, along with the figures in Table 1, it is evident that the hyaluronidase inhibition of the polyanions shows a general pattern which is similar to that of the effects observed in the present investigation. The deviations from the general pattern are few. By measurement of the effect of a polyanion in one biological function it is almost possible to predict the actions in other systems.

Chemically and physically, the present polyanions constitute a very heterogeneous group (see Table 1). They include various types of anionic macromolecules with aromatic and aliphatic carboxylates, primary and secondary phosphates or sulfonates. The substances also include certain groups of polymers which permit a closer comparison between the physicochemical structure (Table 1) and the bio-activities (Table 2).

- The molecular size of the stilbol phosphates (151 A, 116 G, 116 C and 116 F) appears to increase in parallel with the intensity in their different biological actions.
- The amount of acid esters of phthalic acid in dextran (128 A, 129 A and 127 A) is in close correlation with the degree of biological effectiveness of these carboxylate polymers.
- When the primary phosphates in polystilbol phosphate (116 C)

are hydrolyzed off (139 G), there seems to be a corresponding drop in the bio activities

- (d) The addition of basic groups to polyphloretin phosphate (101 A) appears to impair the activity of the polymer (160 A)

TABLE 2

*Comparison of Biological Activities of Heparin and Certain Synthetic Polyanions The Polyanions Are Given According to their Antihyaluronidase Activity*

| Polyanion | Anti thrombin effect* | $\mu\text{g}$ neutralized by 1 $\mu\text{g}$ of lyobrene® | Metachromatic activity | Inhibition of herpes virus† | Haemagglutinating activity‡ | $\mu\text{g}$ ml causing clearing clumping of ileitacells |
|-----------|-----------------------|---|------------------------|-----------------------------|-----------------------------|---|
| Heparin   | 1                     | 11  | +                      | 0.1                         | No effect                   | 3.6   |
| 131 A     | 3.4                   | 1   | +                      | 0.2                         | 0.5                         | 1.5   |
| 137 B     | 2.2                   | 0.4   | +                      | 0.2                         | 1                           | 6   |
| 177 A     | 4.6                   | 2   | +                      | 0.2                         | 1                           | 8   |
| 178 A     | 2.2                   | 1   | +                      | 2.4                         | 1                           | 3   |
| 116 F     | 20 ■                  | 0.5   | +                      | 0.6                         | 0.5                         | 8   |
| 116 C     | 40-50                 | 3   | -?                     | 1.4                         | 1                           | 8   |
| 153 A     | 30-40                 | 3   | +                      | 0.3                         | 2.5                         | 8   |
| 114 C     | 25-30                 | 5   | —                      | 1.1                         | 1                           | 25  |
| 140 A     | 90-90                 | 8   | -?                     | 1.5                         | 10                          | 12  |
| 139 C     | 80-300                | 100   | —                      | 2.3                         | 2.5                         | 12  |
| 155 A     | 6-8                   | 2   | +                      | 1.1                         | 5                           | 25  |
| 116 G     | 45-45                 | 25  | +                      | 2.1                         | 2.5                         | 19  |
| 101 A     | 30-80                 | 5   | +                      | 1.3                         | 25                          | 250   |
| 154 A     | 3.5                   | 25  | ?                      | 1.9                         | 10                          | 250   |
| 159 A     | 80-150                | 17  | —                      | 1.0                         | 1000                        | 50  |
| 142 B     | 60-110                | 10  | ?                      | 1.8                         | 5                           | ■   |
| 130 A     | 3.3                   | 0.3   | —                      | 2.4                         | 50                          | 25  |
| 129 A     | 6-6                   | 1.2   | —                      | 1.0                         | 2500                        | 6   |
| 176 A     | 3.3                   | 0.5   | —                      | 1.8                         | 2500                        | 12  |
| 132 A     | 300-300               | 0.3   | —                      | 40                          | No effect                   | No effect   |
| 128 A     | 25-30                 | 2.5   | —                      | 80                          | No effect                   | No effect   |
| 131 A     | No effect             | —   | —                      | No effect                   | No effect                   | No effect   |
| 164 A     | 50-200                | 12  | —                      | 10                          | No effect                   | No effect   |
| 160 A     | 300-330               | 650   | —                      | No effect                   | No effect                   | No effect   |

The quantity in  $\mu\text{g}$  per ml with the same antithrombin activity as 1  $\mu\text{g}$  heparin per ml

† The quantity in  $\mu\text{g}$  per ml reducing the number of plaques by 50 per cent

‡ Minimum quantity in  $\mu\text{g}$  per ml causing haemagglutination of chicken red cells

• Macroscopic metachromatic staining of toluidine blue with 100  $\mu\text{g}$  per ml of polyanion.

## DISCUSSION

The available evidence presented above seems to indicate that this biological activity of the polyanions may be correlated with the net amount of anionic groups (electronegativity) and the molecular size, to which the anticoagulant activity of heparin (Engelberg 1963) is said to be related.

The so-called heparinoids have thus far been sulphated esters of large molecular compounds mostly of polysaccharides polyuronides and mucopolysaccharides but also of synthetic macromolecules (Pulver 1961). Studies of antithrombin activity and neutralization by Poly-

brene® appear to indicate that the active polyanions investigated and not only sulphated esters, act like heparin, and can thus be termed heparinoids

The metachromatic reaction, which depends primarily on the formation of bonds between the basic amino groups of the dye and the anionic groups of heparin and heparinoids, is said to be reasonably specific in the presence of NaCl *in vitro* (Jaques & Bell 1959). The experiments with toluidine blue further corroborate the heparinoid nature of the present macromolecules.

Nevertheless, marked differences exist between the bio activities of the natural polyanion, heparin and the present synthetic polyanions. The synthetic polyanions had anticoagulant functions in the thrombin-fibrinogen system. Thus they did not need a cofactor, which is a well established pre-requisite for the antithrombin activity of heparin. Secondly, in spite of its potent actions in many biological systems heparin did not agglutinate chicken red cells. Thirdly, it seems that the effect of heparin on cell attachment and growth behaviour is different from that caused by either fresh human sera or the present synthetic polyanions (Nordling *et al* 1964b). Furthermore, investigation on the mechanism of the antiviral action of heparin and other polyanions (Vaheri 1964) has emphasized other, presumably associated divergences. All polyanions inhibited the early interaction of herpes virus and the cell, but the relation between reversible ("dissociable") and irreversible ("virucidal") action on the virus, in this inhibition showed wide variations for the different polyanions. Thus heparin had only reversible action on the virus, many synthetic polyanions were primarily virucidal and certain heparinoids appeared to hold a mid-position. The degree of virucidal action of the polyanions seemed to correlate with their ability to agglutinate red cells (see also Table 2) and their toxicity to cell cultures.

There is evidence that both the primary attachment of viruses to cells (Philipson 1963) and of cells to surface (Nordling *et al* 1964a) might be electrostatic. It is conceivable that heparin and other polyanions by increasing the net negative charge of viruses and cells (Allison & Valentine 1960), might exercise comparable inhibitory effects on the attachment of herpes virus to cells and HeLa cells to a glass surface. It is also possible that the effects may be comparable with the action of heparin and these polyanions on blood platelets. However, since heparin and the present polyanions are strongly inhibitory on herpes virus in saline medium (Vaheri 1964) but apparently do not prevent cell attachment without serum (cofactor or carrier) (Nordling *et al* 1964b), it is likely that the mechanisms differ.

The present investigation emphasizes the broad bio-activities of polyanionic macromolecules. The diverse actions of heparin, and other polyanions can be supposed to derive from their general reactivity with basic sites in many proteins and other substances (Jaques & Bell



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## CHOLERA FILTRATE AND VASOPRESSIN

### *Antagonistic Effect on the Isolated Frog Skin*

By

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Received 8 iv 64

During the past 10 years strong evidence has accumulated that the classical concept of Asiatic cholera is incorrect. The denudation of the intestinal epithelium claimed to be caused by the vibrio was not found on repeated investigations. A new theory postulated an inhibition of the active transport of sodium as the cause of the diarrhoea. Much of this evidence is collected in studies conducted by US Naval Medical Research Units and it is reviewed by Phillips (1963). He also presents evidence of a sodium pump inhibitor in stools of cholera patients.

A similar inhibitor was found by Fuhrman in a commercial cholera filtrate (Fuhrman & Fuhrman 1960, Fuhrman, Fuhrman & Burrows 1962). The preparation used is produced by Philips Duphar as a source of neuraminidase which is an enzyme utilized in influenza diagnosis. When the Cholera Filtrate was brought into contact with the anatomical outside of the isolated frog skin the active transport of sodium was strongly inhibited. No inhibition was found when the filtrate was applied to the inside of the skin.

Several but not all batches of this preparation were inhibitory. Attempts to produce active filtrates from different substrates and with different strains of *V. cholerae* were almost fruitless and only one preparation showed slight inhibition. Endotoxin produced by the method of Westhal proved inactive as well as several other toxin preparations.

The inhibiting factor was dialysable and a molecular weight of a few hundred was suggested. It was negatively charged at neutral pH and was relatively stable to heating, acid and alkali.

### MATERIAL AND METHODS

Experiments were performed mostly with the skin from the *Rana lessonae*.

was equipped with a meter for measuring the potential difference between the two compartments and with a short circuiting device composed of a 108 volt anode battery, a potential divider and a micro-ammeter. This makes it possible to short circuit the frog skin by the application of an external electromotive force, and then to measure the current produced by the skin under these conditions. The current measured in this way is the so called 'short circuit current'. *Ussing & Zerahn* showed that the short circuit current is equal to the net flux of sodium ions when current and flux are expressed in the same units.

During experiments the potential divider was constantly adjusted to maintain the potential difference across the skin at zero. For measuring the potential under normal conditions the anode battery was only momentarily disconnected.

Cholera filtrate was obtained from N. V. Philips Duphar, Amsterdam<sup>1</sup>. It was received freeze dried in ampoules, and each ampoule was dissolved in 10 ml of 'NaCl free frog Ringer solution'. The filtrate contains no bacteriostatic.

Vasopressin was obtained from A/S Alfred Benzon, Copenhagen, under the name 'Insipidin'. It was claimed to contain 20 International Units (IU) per ml of vasopressor substances.

The copper solution was a solution of  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ . The concentration was 625 mg per litre. 0.1 ml of this solution in 25 ml gives a final concentration of  $10^{-5} \text{M}$ .

The anti-cholera serum was a pool from ten rabbits immunized with the International Reference Preparation of Cholera antigen (Inaba).

The frog-Ringer solution was composed as follows:

|                                     |         |
|-------------------------------------|---------|
| $\text{NaHCO}_3$                    | 0.20 g  |
| KCl                                 | 0.20 g  |
| NaCl                                | 6.50 g  |
| $\text{CaCl}_2$ (solution 24 % w/v) | 0.41 ml |
| Redistilled water to                | 1000 ml |

In the 'NaCl free frog Ringer solution' the NaCl was omitted.

## RESULTS

Fig 1, a-d, shows the effect of Cholera Filtrate on the short-circuit current. An inactive filtrate provoked only a slight and reversible rise in current (1 a), whereas the active filtrate caused a rapid, pronounced decrease after an initial rise (1 b). This takes place when the filtrate is added to the outside solution. When the same dose is added to the inside solution only a very slight and transitory fall in current follows (1 c), but contrary to *Fuhrman*, the addition of four times as much to the inside, caused an immediate, precipitous decrease. No initial rise was observed in this case (1 d). Regarding the addition of vasopressin, see below.

As the decrease after external application was irreversible, thus making a proper control experiment on the same skin impossible, we tried to find another control method. A useful tool was found in the neurohypophyseal hormones. It is well-known (*Fuhrman & Ussing* 1961), that these hormones have a decidedly increasing effect on the short-circuit current when they are added to the inside solution. Depending on the doses applied this effect was more or less abolished by Cholera Filtrate.

<sup>1</sup> We are grateful to Dr *Hert-berger*, Philips Duphar, for supplying samples of several batches of Cholera Filtrate.

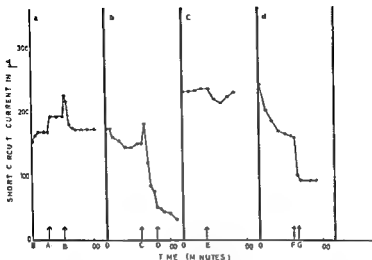


Fig 1  
The effect of Cholera Filtrate

- a A 10 ml inactive Cholera Filtrate to outside
- B Outside solution changed
- b C. 0.5 ml active Cholera Filtrate to outside
- D 0.2 IU vasopressin to inside
- c E 0.5 ml active Cholera Filtrate to inside
- d F 2.0 ml active Cholera Filtrate to inside
- G 0.2 IU vasopressin to inside

The effect of 0.2 IU vasopressin on the normal untreated frog skin, is demonstrated in Fig 2 whereas application of vasopressin after active Cholera Filtrate is shown in Fig 1 b. A close correlation has been observed between the decrease in current and the degree of inhibition of vasopressin effect produced by different doses of the same filtrate. This may be used for quantitative estimation of Cholera Filtrates.

Sometimes the frog skin will never or only very slowly, reach a stable short circuit current. In such cases a stabilization may frequently be obtained by the addition of  $\text{Cu}^{++}$  (Ussing 1955). It is demonstrated in Fig 3 that the skin is still sensitive to Cholera Filtrate after this treatment.

In Fig 3 the potential difference across the skin when the external electromotive force is disconnected is also recorded. As in this experiment we always found the effect of Cholera Filtrate on the potential difference very slight. This disagrees with the findings of Fuhrman.

Even when the skin is not stabilized it is possible to demonstrate the activity of Cholera Filtrate. The effect of vasopressin on such an unstable skin preparation is shown in Fig 4 a and in Fig 4 b the effect of almost simultaneous application of vasopressin and Cholera Filtrate is presented. In the latter case the effect of vasopressin is almost blocked.



was equipped with a meter for measuring the potential difference between the two compartments and with a short circuiting device composed of a 108 volt anode battery, a potential divider and a micro ammeter. This makes it possible to short circuit the frog skin by the application of an external electromotive force, and then to measure the current produced by the skin under these conditions. The current measured in this way is the so called 'short circuit current'. Ussing & Zerahn showed that the short circuit current is equal to the net flux of sodium ions when current and flux are expressed in the same units.

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The frog Ringer solution was composed as follows:

|                                    |         |
|------------------------------------|---------|
| $\text{NaHCO}_3$                   | 0.20 g  |
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| NaCl                               | 6.50 g  |
| $\text{CaCl}_2$ (solution 24% w/v) | 0.41 ml |
| Redistilled water to               | 1000 ml |

In the 'NaCl-free frog Ringer solution' the NaCl was omitted.

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Fig 1, a-d, shows the effect of Cholera Filtrate on the short-circuit current. An inactive filtrate provoked only a slight and reversible rise in current (1 a), whereas the active filtrate caused a rapid, pronounced decrease after an initial rise (1 b). This takes place when the filtrate is added to the outside solution. When the same dose is added to the inside solution only a very slight and transitory fall in current follows (1 c), but contrary to *Fuhrman*, the addition of four times as much to the inside, caused an immediate, precipitous decrease. No initial rise was observed in this case (1 d). Regarding the addition of vasopressin, see below.

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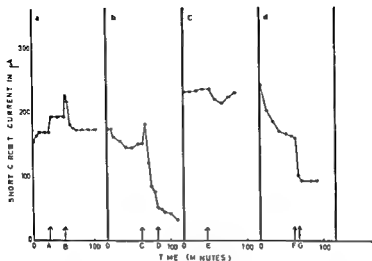


Fig 1

## The effect of Cholera Filtrate

- a A 10 ml inactive Cholera Filtrate to outside  
B Outside solution changed
- b C 0.5 ml active Cholera Filtrate to outside  
D 0.2 I U vasopressin to inside
- c E 0.5 ml active Cholera Filtrate to inside
- d F 2.0 ml active Cholera Filtrate to inside  
G 0.2 I U vasopressin to inside

The effect of 0.2 I U vasopressin on the normal, untreated frog-skin, is demonstrated in Fig 2, whereas application of vasopressin after active Cholera Filtrate is shown in Fig 1 b. A close correlation has been observed between the decrease in current and the degree of inhibition of vasopressin effect produced by different doses of the same filtrate. This may be used for quantitative estimation of Cholera Filtrates.

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The copper solution was a solution of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . The concentration was 6.75 mg per litre. 0.1 ml of this solution in 25 ml gives a final concentration of  $10^{-4}\text{M}$ .

The anti cholera serum was a pool from ten rabbits immunized with the International Reference Preparation of Cholera antigen (Inaba).

The frog Ringer solution was composed as follows:

|                                     |         |
|-------------------------------------|---------|
| $\text{NaHCO}_3$                    | 0.20 g  |
| KCl                                 | 0.20 g  |
| NaCl                                | 6.50 g  |
| $\text{CaCl}_2$ (solution 24 % w/v) | 0.41 ml |
| Redistilled water to                | 1000 ml |

In the NaCl free frog Ringer solution the NaCl was omitted.

## RESULTS

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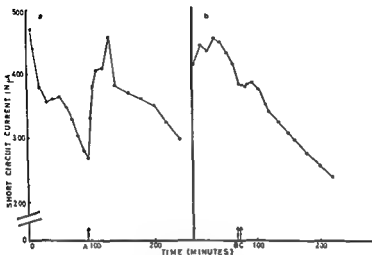


Fig 4

The effect of vasopressin and Cholera Filtrate on unstable skin

- a A 0.2 IU vasopressin to inside
- b B 1 IU vasopressin to inside
- C 0.5 ml active Cholera Filtrate to outside

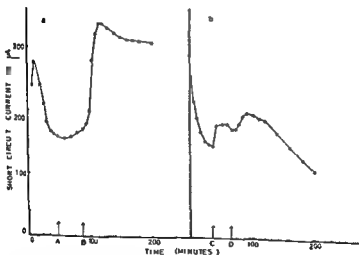


Fig 5

The effect of Anti Cholera Serum

- a A 0.1 ml Anti Cholera Serum to outside
- B 0.2 IU vasopressin to inside
- b C 0.5 ml active Cholera Filtrate incubated for 19 hours at 4° C with 0.1 ml Anti Cholera Serum to outside
- 0.2 IU vasopressin to inside

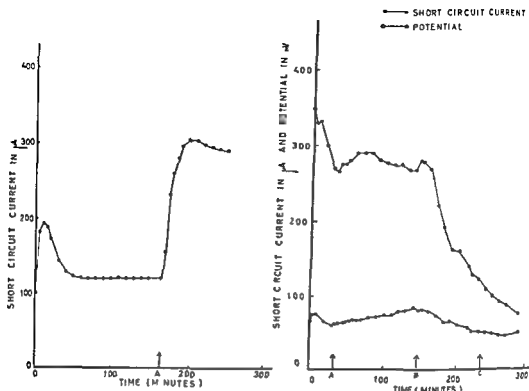


Fig 2

Fig 2

The effect of vasopressin on the frog skin  
A 0.2 I.U. vasopressin to inside

Fig 3

The effect of Cholera Filtrate on the Cu<sup>++</sup>-treated skin  
A 0.1 ml CuSO<sub>4</sub> solution to outside  
B 0.5 ml active Cholera Filtrate to outside  
C 0.1 ml Anti Cholera Serum to outside

It was also found of interest to see whether an anti cholera serum had any effect on the response. In Fig 5a the addition of 0.1 ml of serum had no effect on the short-circuit current or on the ability of the skin to react to vasopressin. In Fig 3 the same dose could not reverse the effect of Cholera Filtrate added previously. In Fig 5b, however, the skin was treated with 0.5 ml of Cholera Filtrate, previously incubated with 0.1 ml of serum. The inhibitory effect on the current was effected whereas the vasopressin response was only partially inhibited.

## DISCUSSION

The theory that the effect of the vibrios in Asiatic cholera is to inhibit the active transport of sodium from the lumen of the intestine to the blood, looked very promising to us. This paper describes experiments confirming earlier observations made by Fuhrman about the

The possibility is mentioned for utilizing this antagonism for a quantitative estimate of the frog skin paralyzing substance

It is demonstrated that incubation of the Cholera Filtrate with an anti cholera serum prior to addition to the frog skin inhibits the paralysis

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presence of a substance in 'Cholera Filtrate, Philips-Duphar' inhibiting the short-circuit current in isolated frog-skin

It is confirmed that when the Cholera Filtrate is added to the outside of the skin, a rise in current will occur initially, but after 15-20 minutes the current will start to decrease. The steepness and size of this decrease seem to be dependent on the dose of Cholera Filtrate. *Fuhrman* found that Cholera Filtrate had no such effect when added to the anatomical inside of the frog skin. We, however, have shown that an inhibitory effect is also present in that case, but with a somewhat lower sensitivity. The initial increase seen after external application did not occur after internal application. This may be explained by the presence of the 'sodium-pump' near the inside surface of the skin. If the response is the result of a competition between an unspecific stimulation of the skin (*e.g.* a nutritional effect) and the inhibition of the 'pump', then it will take some time for the inhibitor to reach the point of attack from the outside. During this time the stimulatory effect will dominate. The difference in sensitivity of the two surfaces is not so easily explained.

Because of the irreversibility of the Cholera Filtrate response, and because unspecific decreases or altogether unstable skins were sometimes encountered, the vasopressin effect was investigated. This 'check' has been very useful, although it will not distinguish between an unspecific metabolic inhibition and a specific inhibition of the 'sodium pump'. The antagonistic effect of vasopressin may be used for quantitative estimation of the inhibitor but due to the slowness of the reaction such an estimation will be rather laborious.

The reaction between the inhibitory factor and anti-cholera serum has not been very thoroughly investigated because of shortage of active Cholera Filtrate. In the experiment described in Fig 5b, perhaps the vasopressin was added too early. A modification of the response is, however, quite obvious. It should be mentioned that the serum used was prepared by means of antigen from the Inaba type of *V. cholerae*, whereas the strain 4Z, used for the production of the Cholera Filtrate, belongs to the Ogawa serotype.

We have not investigated the nature of the inhibitor very closely this being due to shortage of material also. We have, however, been able to confirm that it is dialysable and, at least to some degree, heat-stable.

#### SUMMARY

*Fuhrman's* discovery is confirmed that 'Cholera Filtrate, Philips-Duphar' may contain a heat-stable, dialysable frog-skin paralyzing substance.

An antagonistic effect between cholera filtrate and vasopressin on the frog-skin is demonstrated. It is found also to be effective on the Cu<sup>++</sup>-treated skin.

inactivation than syphilitic reactions (1) This constituted an additional reason for recommending heat inactivation However by a thorough study of this problem it was demonstrated that the heat lability of the reaction was by no means a safe criterion for distinguishing non syphilitic from syphilitic reactions (7)

The introduction of the purified phospholipid antigen cardiolipin (9) gave a much better defined antigen for the Wassermann complement fixation test but did not alter the significance or the side effects of preliminary heat inactivation of serum (2 12)

According to *Schmidt* (12) heat inactivation usually results in a reduction in the titre of both syphilitic and non syphilitic antisera the syphilitic generally being the more heat stable Inactivation of native complement would be expected to reduce haemolysis thus leading to higher titres being recorded Since the reverse is usually the case changes tending to enhance haemolysis seem to be the predominant result of heat inactivation

The reduction in titre registered after heat inactivation might be due either to characteristics of the antilipoidal immune globulins which themselves may be susceptible to thermal influences or to other serum components whose influence on the complement fixation test changes with heat inactivation

The purpose of the present study is to investigate the influence of various serum components on the outcome of the Wassermann complement fixation test in reactive syphilitic sera as well as the variations in this influence after heat inactivation

## MATERIAL AND METHODS

### *Serum and Cerebrospinal Fluid (CSF)*

TABLE 1

pc

he

C<sub>50</sub>

### *Wassermann Complement Fixation Test with Cardiolipin Antigen*

The standard procedure in this laboratory is Morch's modification of the original Wassermann test using cardiolipin antigen This technique has been described in detail by *Schmidt* (10)

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40. 41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60. 61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80. 81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100. 101. 102. 103. 104. 105. 106. 107. 108. 109. 110. 111. 112. 113. 114. 115. 116. 117. 118. 119. 120. 121. 122. 123. 124. 125. 126. 127. 128. 129. 130. 131. 132. 133. 134. 135. 136. 137. 138. 139. 140. 141. 142. 143. 144. 145. 146. 147. 148. 149. 150. 151. 152. 153. 154. 155. 156. 157. 158. 159. 160. 161. 162. 163. 164. 165. 166. 167. 168. 169. 170. 171. 172. 173. 174. 175. 176. 177. 178. 179. 180. 181. 182. 183. 184. 185. 186. 187. 188. 189. 190. 191. 192. 193. 194. 195. 196. 197. 198. 199. 200. 201. 202. 203. 204. 205. 206. 207. 208. 209. 210. 211. 212. 213. 214. 215. 216. 217. 218. 219. 220. 221. 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## STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

### *1 Influence of Normal Components of Human Serum and Cerebrospinal Fluid on the Outcome of the Complement Fixation Test with Cardiolipin Antigen, and Variation in this Influence Following Heat Inactivation*

By

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Most serological examinations in complement fixation tests are preceded by thermal conditioning of the serum, called heat inactivation, usually consisting of 30 minutes' incubation of serum samples in open tubes on a waterbath maintained at a constant temperature of 56° C.

This procedure was originally introduced in order to inactivate native human complement which might otherwise supplement to varying extents the well-defined amount of guinea-pig complement added in the complement fixation test.

Heat inactivation results in irreversible destruction of the thermolabile complement factors C'1 and C'2. The other complement factors C'3a, C'3b and C'4 are more resistant to heat but also suffer partial or total loss of activity during the usual heat inactivation procedure (5).

Although the wish to inactivate human complement was the theoretical background for using heat inactivation of the serum as a standard procedure prior to examination in the Wassermann complement fixation test for detection of antilipoidal immune globulins in syphilis, it soon became obvious that this was far from being its only effect.

A number of sera give more conclusive results after heat inactivation. This has been ascribed to a destruction of thermolabile native haemolysins during heat inactivation (3) and is also due to the fact that a number of sera which are slightly anticomplementary in the unheated state fail to show anticomplementary activity after heat inactivation. On the other hand, some sera—especially from cases of paraproteinæmia—may develop a strongly anticomplementary activity after heat inactivation, without showing any anticomplementary activity in the unheated state (8). Some sera show reactivity before but not after heat inactivation (13), and many give higher titres before than after heat inactivation (6). However, with some sera the reverse was the case (3).

Non-syphilitic reactions in the Wassermann complement fixation test with the original crude lipid antigen seemed more susceptible to heat

inactivation than syphilitic reactions (1). This constituted an additional reason for recommending heat inactivation. However by a thorough study of this problem, it was demonstrated that the heat lability of the reaction was by no means a safe criterion for distinguishing non syphilitic from syphilitic reactions (7).

The introduction of the purified phospholipid antigen cardiolipin (9) gave a much better defined antigen for the Wassermann complement fixation test but did not alter the significance or the side effects of preliminary heat inactivation of serum (2, 12).

According to Schmidt (12), heat inactivation usually results in a reduction in the titre of both syphilitic and non syphilitic antisera, the syphilitic generally being the more heat stable. Inactivation of native complement would be expected to reduce haemolysis thus leading to higher titres being recorded. Since the reverse is usually the case, changes tending to enhance haemolysis seem to be the predominant result of heat inactivation.

The reduction in titre registered after heat inactivation might be due either to characteristics of the antilipoidal immune globulins which themselves may be susceptible to thermal influences or to other serum components whose influence on the complement fixation test changes with heat inactivation.

The purpose of the present study is to investigate the influence of various serum components on the outcome of the Wassermann complement fixation test in reactive syphilitic sera, as well as the variations in this influence after heat inactivation.

## MATERIAL AND METHODS

### *Serum and Cerebrospinal Fluid (CSF)*

The 3

### *Wassermann Complement Fixation Test with Cardiolipin Antigen*

The standard procedure in this laboratory is Morch's modification of the original Wassermann test using cardiolipin antigen. This technique has been described in detail by Schmidt (10).

Complement fixation process is allowed two hours at 4° C and 30 minutes at

37° C before the addition of sheep blood (25 per cent suspension in saline) and amboceptor

Haemolysis is read after 1 hour at 37° C and 18 hours at 4° C by comparison with a colour scale prepared each day. Titres are expressed in  $\log_{10}$  values of the dilutions giving 50 per cent haemolysis calculated according to the Kärber method (4)

The guinea pig complement titre is determined every other day against amboceptor and sheep blood. The amboceptor titre is kept constant for longer intervals (6 months)

The antigen consists of 0.0175 per cent (w/v) cardiolipin, 0.0875 per cent (w/v) egg lecithin (both prepared at the Statens Seruminstitut), and 0.3 per cent (w/v) cholesterol (Pfanstiehl) in dehydrated ethanol

Examination of serum for content of native human complement was performed in the same way as the quantitative Wassermann reaction, except that the addition of guinea pig complement was omitted

### EXPERIMENTAL DATA

1 As it can be seen from the description of our method, the quantitative determination of the antilipoidal immune globulins in the Wassermann complement fixation test is accomplished by performing a serial three fold dilution of serum in physiological saline

This means that the titration end-point is determined in the presence of higher concentrations of other serum components for weak antisera than for antisera with high titres

In order to eliminate this difference the quantitative determination of antilipoidal immune globulins in eight syphilitic sera was performed by dilution of antiserum in unheated and in heat inactivated non-reactive serum pool from healthy human donors instead of in saline

TABLE 1

*Quantitative Examination of Eight Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen. Comparison Between Serial Dilutions in Saline, Unheated and Heat Inactivated Non Syphilitic Serum*

| Serum no                                       | Diagnosis | Dilution<br>in saline<br>$\log_{10}$ value | Dilution<br>in unheated<br>non reactive<br>serum pool<br>$\log_{10}$ value | Dilution<br>in inactivated<br>non reactive<br>serum pool<br>$\log_{10}$ value |
|--|-----------|--|--|---|
| 179  | S I       | 1.193                                      | 0.239  | 0.930   |
| 193  | S I       | 0.692                                      | 0.239  | 0.334   |
| 182  | S II      | 0.692                                      | 0.239  | 0.296   |
| 184  | S II      | 1.431                                      | 1.026  | 1.193   |
| 192  | S II      | 1.431                                      | 0.811  | 1.145   |
| 180  | S lat rec | 0.907                                      | 0.296  | 0.334   |
| 183  | S lat rec | 0.716                                      | 0.239  | 0.239   |
| 189  | S lat rec | 0.620                                      | 0.239  | 0.239   |
| Mean $\log_{10}$ value                         |           | 0.960                                      | 0.415  | 0.588   |
| Mean reduction in titre                        |           |  | 0.545  | 0.372   |
| Mean reduction in titre (unheated/inactivated) |           |  |  | 0.173   |
| Standard error                                 |           |  | 0.07   |   |

A comparison of the results with those of the standard procedure with dilution in saline performed simultaneously is shown in Table 1

It will be seen from Table 1 that dilution in normal human serum pool, both unheated and heat inactivated, reduces the reactivity of syphilitic sera compared with the reactivity registered after dilution in saline. Dilution in unheated serum pool results in a greater reduction in reactivity than dilution in heat inactivated serum pool, a fact which might be explained by the presence of native complement in the former.

However, this experiment demonstrates that other serum components besides the native complement play a rôle in reducing the reactivity. Otherwise it would be impossible to explain the reduction caused by the heat inactivated serum pool. Serum components which inhibit complement fixation have been demonstrated earlier (7, 11) and their possible mode of action has been discussed (14).

2. The total amount of serum, undiluted or diluted, in the non reactive serum pool in the first experiments was 0.020–0.060 ml in each tube according to the standard technique described.

In order to bring out more clearly the influence of the components of the non reactive serum pool, the heat inactivated sera examined in our second experiment were diluted serially in saline, after which 0.3 ml of unheated non reactive serum pool was added to each tube before the addition of guinea pig complement and cardiolipin antigen.

The effect of adding 0.3 ml of heat inactivated serum pool and 0.3 ml of saline to each tube was determined in the same manner.

TABLE 2

*Quantitative Examination of Eight Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen in the Presence of 0.3 ml of Saline Unheated or Heat Inactivated Non Syphilitic Serum Added Prior to Complement Fixation*  
Results Given in log<sub>10</sub> Values

| Serum no                                       | Diagnosis | 1:1 titration to each tube of 0.3 ml of |                                  |                                |
|--|-----------|---|----------------------------------|--------------------------------|
|  |           | Saline                                  | Unheated non reactive serum pool | Heated non reactive serum pool |
| 179  | S I       | 1.288                                   | 0.000                            | 0.573                          |
| 193  | S I       | 0.740                                   | 0.000                            | 0.310                          |
| 182  | S II      | 0.787                                   | 0.000                            | 0.239                          |
| 184  | S II      | 1.336                                   | 0.000                            | 0.763                          |
| 191  | S II      | 1.422                                   | 0.000                            | 0.763                          |
| 180  | S lat rec | 0.811                                   | 0.000                            | 0.239                          |
| 183  | S lat rec | 0.763                                   | 0.000                            | 0.191                          |
| 189  | S lat rec | 0.620                                   | 0.000                            | 0.239                          |
| Mean log <sub>10</sub> value                   |           | 0.975                                   | 0.000                            | 0.415                          |
| Mean reduction in titre                        |           |   | (0.975)                          | 0.560                          |
| Mean reduction in titre (unheated inactivated) |           |   |                                  | (— 0.415)                      |
| Standard error                                 |           |   |                                  | 0.057                          |

Table 2 shows the result of these experiments, all of which were performed on the same day

It will be seen from Table 2 as compared with Table 1 that the addition of 0.3 ml of saline to the volume in which the complement fixation takes place (0.3 ml) did not influence the result. Therefore the reduction in titre registered after the addition of 0.3 ml of non-reactive serum pool must be due, not to changes in the reaction volume, but to normal human serum components.

As might be expected, the reduction in titre is more pronounced than in the first two experiments, the influence of unheated serum pool again exceeding that of heat inactivated serum pool.

3. In order to characterize the serum factors responsible for the decrease in reactivity registered on titration in heat inactivated human non-reactive serum pool, a study of the influence of various serum proteins on the outcome of the complement fixation test was performed.

The various serum proteins were salted out by dialysis against solutions of ammonium sulphate of varying concentrations. The protein which was precipitated with 40 per cent saturation was designated gamma-globulin, that precipitated with 50 per cent saturation alpha and beta-globulin, and that precipitated with 68 per cent saturation albumin.

The precipitates were redissolved in saline and dialysed against buffered saline. Then a number of syphilitic sera were titrated in each of the three protein solutions as well as in the original non-reactive serum pool from which the serum protein fractions were derived.

After dialysis, the heat inactivated serum protein fractions used in this experiment showed no anticomplementary activity in the doses used, i.e. 0.060 ml at most.

TABLE 3

*Quantitative Examination of Five Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen. Comparison between Serial Dilutions in Saline, Whole Non Syphilitic Serum and Three Heat Inactivated Solutions of Fractionated Non Syphilitic Serum Proteins. Results given in log<sub>10</sub> values*

| Serum no                     | Diagnosis  | Diluted in saline | Diluted in heat inactivated non reactive |        |            |         |
|------------------------------|------------|-------------------|--|--------|------------|---------|
|                              |            |                   | Whole serum                              | γ glob | α & β glob | Albumin |
| 241                          | S I        | 1 002             | 0 525                                    | 0 716  | 0 811      | 0 716   |
| 251                          | S I II     | 1 431             | 1 026                                    | 1 097  | 1 288      | 1 241   |
| 243                          | S II       | 1 241             | 0 978                                    | 1 097  | 1 312      | 1 145   |
| 276                          | S II       | 1 861             | 1 169                                    | 1 312  | 1 527      | 1 575   |
| 261                          | S lat tard | 1 193             | 1 121                                    | 1 217  | 1 241      | 1 193   |
| Mean log <sub>10</sub> value |            | 1 346             | 0 964                                    | 1 088  | 1 236      | 1 174   |
| Mean reduction in titre      |            |                   | 0 382                                    | 0 258  | 0 110      | 0 172   |
| Standard error               |            |                   |  | 0 073  |            |         |

An attempt to add excessive amounts of the individual serum protein fractions to each tube in the titration series was unsuccessful due to the anticomplementary action in high doses of these fractions.

It will be seen from Table 3 that each of the protein fractions is able to reduce the titre of syphilitic sera under the conditions of these experiments. The variations in their reducing capacity are not significant, although it would appear that the serum proteins precipitated with 40 per cent saturated ammonium sulphate contain most of the reducing capacity demonstrated in whole serum. Inhibitors have been demonstrated previously in the albumin fraction precipitated with ammonium sulphate (7) and in the albumin + alpha 1 fraction of Tiselius electrophoresis (11).

4 Since cerebrospinal fluid does not contain complement, heat inactivation is not considered necessary for such samples before complement fixation testing. It is also found that the reactivity of cerebrospinal fluid is influenced only slightly by the standard heat inactivation at 50° C for 30 minutes.

On this background it has been considered worth while to examine the influence of non reactive cerebrospinal fluid on the reactivity of antilipoidal reactive syphilitic sera.

The effect of serial dilution in non reactive CSF instead of in saline is shown in Table 4.

TABLE 4

*Quantitative Examination of 10 Heat Inactivated Syphilitic Sera in Complement Fixation with Carlistipin Antigen. Comparison between Serial Dilutions in Saline, Unheated and Heat Inactivated Non Syphilitic Cerebrospinal Fluid (CSF). Results Given in log<sub>10</sub> Values*

| Serum no   | Diagnosis  | Original dilution in saline | Dilute 1 in                   |                                 |
|--|------------|-----------------------------|-------------------------------|---------------------------------|
|  |            |                             | Unheated non-reactive 1% pool | Heat inact non-reactive 1% pool |
| 303  | S I        | 0.907                       | 0.859                         | 0.924                           |
| 310  | S I        | 1.431                       | 1.288                         | 1.241                           |
| 306  | S II       | 1.020                       | 0.859                         | 0.930                           |
| 307  | S II       | 1.193                       | 0.907                         | 0.829                           |
| 318  | S lat rec  | 1.241                       | 1.288                         | 1.193                           |
| 319  | S lat rec  | 0.262                       | 0.310                         | 0.286                           |
| 299  | S lat tard | 0.930                       | 0.710                         | 0.811                           |
| 298  | S III      | 1.622                       | 1.479                         | 1.479                           |
| 300  | S III      | 2.147                       | 1.622                         | 1.431                           |
| 301  | S III      | 1.479                       | 1.193                         | 1.288                           |
| Mean log <sub>10</sub> value                         |            | 1.226                       | 1.055                         | 1.047                           |
| Mean reduction in titre (compared to original titre) |            |                             | 0.171                         | 0.179                           |
| Mean reduction in titre (unheated inactivated)       |            |                             |                               | 0.008                           |
| Standard error                                       |            |                             |                               | 0.051                           |

Table 2 shows the result of these experiments, all of which were performed on the same day.

It will be seen from Table 2 as compared with Table 1 that the addition of 0.3 ml of saline to the volume in which the complement fixation takes place (0.3 ml) did not influence the result. Therefore the reduction in titre registered after the addition of 0.3 ml of non-reactive serum pool must be due, not to changes in the reaction volume, but to normal human serum components.

As might be expected, the reduction in titre is more pronounced than in the first two experiments, the influence of unheated serum pool again exceeding that of heat inactivated serum pool.

3 In order to characterize the serum factors responsible for the decrease in reactivity registered on titration in heat inactivated human non-reactive serum pool, a study of the influence of various serum proteins on the outcome of the complement fixation test was performed.

The various serum proteins were salted out by dialysis against solutions of ammonium sulphate of varying concentrations. The protein which was precipitated with 40 per cent saturation was designated gamma-globulin, that precipitated with 50 per cent saturation alpha and beta-globulin, and that precipitated with 68 per cent saturation albumin.

The precipitates were redissolved in saline and dialysed against buffered saline. Then a number of syphilitic sera were titrated in each of the three protein solutions as well as in the original non-reactive serum pool from which the serum protein fractions were derived.

After dialysis, the heat inactivated serum protein fractions used in this experiment showed no anticomplementary activity in the doses used, i.e. 0.060 ml at most.

TABLE 3

*Quantitative Examination of Five Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen. Comparison between Serial Dilutions in Saline, Whole Non-Syphilitic Serum and Three Heat Inactivated Solutions of Fractionated Non-Syphilitic Serum Proteins. Results given in log<sub>10</sub> values.*

| Serum no                     | Diagnosis  | Diluted in saline | Diluted in heat inactivated non reactive |        |             |         |
|------------------------------|------------|-------------------|--|--------|-------------|---------|
|                              |            |                   | Whole serum                              | γ glob | α og β glob | Albumin |
| 241                          | S I        | 1 002             | 0 525                                    | 0 716  | 0 811       | 0 716   |
| 251                          | S I II     | 1 431             | 1 026                                    | 1 097  | 1 288       | 1 241   |
| 243                          | S II       | 1 241             | 0 978                                    | 1 097  | 1 312       | 1 145   |
| 276                          | S II       | 1 861             | 1 169                                    | 1 312  | 1 527       | 1 575   |
| 261                          | S lat tard | 1 193             | 1 121                                    | 1 217  | 1 241       | 1 193   |
| Mean log <sub>10</sub> value |            | 1 346             | 0 964                                    | 1 088  | 1 236       | 1 174   |
| Mean reduction in titre      |            | —                 | 0 382                                    | 0 258  | 0 110       | 0 172   |
| Standard error               |            |                   |  | 0 073  |             |         |

TABLE 6  
*Examination of Ten Non Syphilitic Sera for Content of Native Complement within  
 24 Hours after Withdrawal of Blood Sample and after Storage at both 3° C and 20° C*

| Serum n                                 | Haemolysis<br>with in 24 hours |       |                                | Haemolysis<br>after 3 days at 3° C |       |                                | Haemolysis<br>after a further 14 days at 20° |     |       |     |      |      |
|---|--------------------------------|-------|--------------------------------|------------------------------------|-------|--------------------------------|--|-----|-------|-----|------|------|
|   | Control                        | 1:1   | Serum dilution<br>1:3 1:9 1:27 | Control                            | 1:1   | Serum dilution<br>1:3 1:9 1:27 | Control                                      | 1:1 | 1:3   | 1:9 | 1:27 | 1:27 |
| b 2542                                  | 100                            | 100   | 5                              | 100                                | 90    | 30 0 0                         | 100  | 100 | 50    | 5   | 0    | 0    |
| b 2543                                  | 100                            | 100   | 0                              | 100                                | 100   | 60 0 0                         | 100  | 100 | 50    | 5   | 0    | 0    |
| b 2545                                  | 100                            | 100   | 0                              | 100                                | 100   | 20 0 0                         | 100  | 100 | 50    | 0   | 0    | 0    |
| b 2548                                  | 100                            | 100   | 10                             | 100                                | 100   | 30 0 0                         | 100  | 100 | 70    | 5   | 0    | 0    |
| b 2549                                  | 100                            | 100   | 5                              | 100                                | 100   | 50 0 0                         | 100  | 100 | 40    | 0   | 0    | 0    |
| b 2550                                  | 100                            | 100   | 5                              | 100                                | 100   | 90 0 0                         | 100  | 100 | 90    | 5   | 0    | 0    |
| b 2551                                  | 100                            | 100   | 0                              | 100                                | 100   | 50 0 0                         | 100  | 100 | 70    | 0   | 0    | 0    |
| b 2552                                  | 100                            | 100   | 20                             | 100                                | 100   | 70 0 0                         | 100  | 100 | 80    | 5   | 0    | 0    |
| b 2553                                  | 100                            | 100   | 0                              | 100                                | 100   | 50 0 0                         | 100  | 100 | 30    | 0   | 0    | 0    |
| b 2559                                  | 100                            | 100   | 0                              | 100                                | 100   | 50 0 0                         | 100  | 100 | 70    | 5   | 0    | 0    |
| Mean %<br>haemolysis                    | 100                            | 80    | 5                              | 100                                | 100   | 50 0 0                         | 100  | 100 | 70    | 5   | 0    | 0    |
| % of haemolysis<br>titre in 1 min value |                                | 0.643 |                                |                                    | 0.477 |                                |  |     | 0.549 |     |      |      |



It will be seen that titration in CSF affects the reactivity measured in the Wasserman complement fixation test. However, there is no appreciable difference in this respect between unheated and heat inactivated CSF.

In order to get a clearer picture of the influence of non-reactive CSF pools on the reaction, the experiment was repeated, this time adding 0.3 ml of unheated CSF pool to each tube in one titration row and 0.3 ml of heat inactivated CSF pool to another titration row of the same sera. In a control series, 0.3 ml of saline was added to each tube (see Table 5).

TABLE 5

*Quantitative Examination of Ten Heat Inactivated Syphilitic Sera in Complement Fixation Test with Cardiolipin Antigen in the Presence of 0.3 ml of Saline, Unheated or Heat Inactivated Cerebrospinal Fluid (CSF) Added Prior to Complement Fixation*  
Results Given in  $\log_{10}$  Values

| Serum no                                       | Diagnosis  | Addition to each tube of 0.3 ml of |                   |                           |
|--|------------|------------------------------------|-------------------|---------------------------|
|  |            | Saline                             | Unheated CSF pool | Heat inactivated CSF pool |
| 303  | S I        | 0.763                              | 0.716             | 0.668                     |
| 310  | S I        | 1.336                              | 1.145             | 0.811                     |
| 306  | S II       | 0.907                              | 0.954             | 0.477                     |
| 307  | S II       | 0.859                              | 0.811             | 0.477                     |
| 318  | S lat rec  | 1.575                              | 1.193             | 0.763                     |
| 319  | S lat rec  | 0.571                              | 0.310             | 0.095                     |
| 299  | S lat tard | 1.097                              | 0.716             | 0.239                     |
| 298  | S III      | 1.527                              | 1.336             | 1.050                     |
| 300  | S III      | 1.670                              | 1.336             | 1.097                     |
| 301  | S III      | 1.431                              | 1.145             | 0.954                     |
| Mean $\log_{10}$ value                         |            | 1.174                              | 0.966             | 0.663                     |
| Mean reduction in titre                        |            |                                    | 0.208             | 0.511                     |
| Mean reduction in titre (unheated/inactivated) |            |                                    | 0.303             |                           |
| Standard error                                 |            |                                    | 0.031             |                           |

It was found from this experiment that there is a difference between the effect of unheated and heat inactivated cerebrospinal fluid pool.

The reduction in titre registered on addition of 0.3 ml of CSF pool to each tube is most pronounced when heat inactivated CSF pool is used. This finding is in contrast to what was found with the serum pool in the previous experiments.

This experiment shows that human proteins as found in the CSF, may develop during heat inactivation increased ability to reduce the complement fixing activity of a given serum. The presence of native complement in serum makes it difficult to ascertain whether the serum proteins behave in the same way.

tion upon such sera is therefore unrelated to the inactivation of native complement

TABLE 7  
*Examination of Ten Non Syphilitic Sera for Content of Native Complement after Storage at 4° C*

| Serum no  | * % haemolysis after 3 days at 4° C |     |                |     |      | * % haemolysis after 17 days at 4° C |             |     |
|---|-------------------------------------|-----|----------------|-----|------|--------------------------------------|-------------|-----|
|   | Control                             | 1/1 | Serum dilut on |     |      | Control                              | Serum dilut |     |
|   |                                     |     | 1/3            | 1/9 | 1/27 |                                      | 1/1         | 1/3 |
| b 2021  | 100                                 | 90  | 20             | 0   | 0    | anticomplementary                    |             |     |
| b 2022  | 100                                 | 90  | 20             | 0   | 0    | 100                                  | 40          | 0   |
| b 2023  | 100                                 | 100 | 20             | 0   | 0    | 100                                  | 30          | 0   |
| b 2024  | 100                                 | 90  | 10             | 0   | 0    | 90                                   | 0           | 0   |
| b 2575  | 100                                 | 90  | 30             | 0   | 0    | 100                                  | 50          | 0   |
| b 2026  | 100                                 | 100 | 50             | 0   | 0    | anticomplementary                    |             |     |
| b 2027  | 100                                 | 100 | 40             | 0   | 0    | anticomplementary                    |             |     |
| b 2028  | 100                                 | 90  | 20             | 0   | 0    | 100                                  | 20          | 0   |
| b 2029  | 100                                 | 100 | 90             | 0   | 0    | 100                                  | 50          | 0   |
| b 2030  | 100                                 | 100 | 30             | 0   | 0    | anticomplementary                    |             |     |
| Mean % haemolysis                               | 100                                 | 100 | 30             | 0   | 0    | 100                                  | 30          | 0   |
| 50% haemolysis titre in log <sub>10</sub> value |                                     |     | 0.381          |     |      |                                      |             |     |

## SUMMARY AND CONCLUSION

1 a Quantitative determination of antilipoidal immune globulins in syphilitic serum was performed by serial dilution in an unheated non-reactive serum pool instead of saline. This resulted in an overall reduction in reactivity as determined from the haemolysis curve

1 b Serial dilution in heat inactivated non reactive serum pool of the same syphilitic sera resulted in a reduction in reactivity which was less pronounced than that following serial dilution in unheated serum pool

2 The addition of 0.3 ml of unheated non reactive serum pool to tube in the serial dilution prior to complement fixation resulted in complete depression of reactivity, i.e. gave full haemolysis in all tubes

3 The addition of 0.3 ml of heat inactivated non reactive serum pool in a similar way resulted in a reduction but not in complete loss of reactivity

4 Serial dilution in heat inactivated solutions of salted out serum protein fractions from a non-reactive human serum pool indicated that the gamma globulin fraction was the most potent in reducing the complement fixing activity of syphilitic antilipoidal immune globulins. The albumin fraction also reduced the reactivity, but its influence was less pronounced

5 The activity of native complement in human serum examined in titration experiments with a number of non reactive sera was only

5 The rôle of native human complement has been elucidated by an experiment in which 10 unheated non-reactive sera were subjected to a quantitative complement fixation test without the addition of guinea pig complement. The first examination of the sera took place within 24 hours of the withdrawal of the blood.

The experiment was repeated three days later, the sera having been stored at 4° C, and again after a further 14 days during which the sera were stored at -20° C. The result of these experiments are shown in Table 6.

It will be seen from Table 6 that only the undiluted and the three times diluted serum contained sufficient amounts of native complement to produce appreciable haemolysis in the absence of guinea-pig complement. The control experiments show that storage (corresponding quite closely to the usual handling of serum received for these experiments) does not influence the complement activity of the unheated sera to any extent.

In order to determine the influence of heat inactivation on this native complement activity, the non reactive human serum pool used in this experiment was examined for complement activity before and after heat inactivation.

| % haemolysis<br>before inactivation |                |    |    |     | % haemolysis<br>after inactivation |                |    |    |     |
|-------------------------------------|----------------|----|----|-----|------------------------------------|----------------|----|----|-----|
| Control                             | Serum dilution |    |    |     | Control                            | Serum dilution |    |    |     |
|                                     | 11             | 13 | 19 | 12* |                                    | 11             | 13 | 19 | 12* |
| 100                                 | 100            | 50 | 0  | 0   | 100                                | 0              | 0  | 0  | 0   |

If storage at 4° C is prolonged for another 14 days, a marked decrease in the complement activity is registered in some sera, while others become anticomplementary (Table 7).

It may be concluded that the native human complement, as normally found in unheated serum, only affects the outcome of the complement fixation test in the first and second tube of the quantitative Wassermann reaction as carried out in this laboratory.

In these two tubes the presence of native complement may result in haemolysis, even though the guinea-pig complement added in the usual Wassermann technique is completely fixed by an antigen/antibody complex.

The fact that the presence of native complement in unheated serum only affects the first two tubes in the quantitative determination of complement fixing antilipoidal immune globulins means that strong antisera may show complete inhibition of haemolysis in the first two tubes, in spite of the presence of both native and guinea pig complement. The titre found in such cases is thus entirely unaffected by the presence of native complement in unheated serum. The influence of heat inactivation

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## STUDIES ON ANTILIPOIDAL IMMUNE GLOBULINS

### 2 Reactivity in Lepromatous Leprosy with a Lecithin free Cardiolipin Antigen (Cardchol) after Ordinary and Prolonged Heat Inactivation

By

FRIK HOLST

Received 8.11.64

Complement fixing antilipoidal immune globulins which react with both the original crude and the more recently purified phospholipoid antigens have been a common finding in patients suffering from leprosy, especially of the malignant lepromatous type (16)

Up to 1949 it was impossible to say with any degree of certainty whether these reactions were due to concomitant treponemal infection.

The introduction of tests for the detection of more specific antibodies, e.g. the *Treponema pallidum* immobilization tests (9) has made it possible to decide with great accuracy which leprosy patients have suffered from a treponemal infection which might explain the presence of antilipoidal immune globulins. The literature on this subject has been reviewed by *Fromm et al* (16)

Most investigators have demonstrated that although a number of those leprosy sera reacting with lipid antigens also reacted in the TPI test there still remained some sera from patients with lepromatous leprosy which reacted with lipid antigens but showed no reaction in the TPI test.

In one study however the investigator was able to conclude that 95 per cent of the antilipoidal reactions could be ascribed to treponemal infection. In that study the reactivity in the TPI test was extremely high (30 per cent) (2)

Previous investigations in this laboratory (13, 14) have shown that a number of TPI non reactive sera from patients with lepromatous leprosy reacted in a complement fixation test with ordinary cardiolipin antigen containing lecithin (CLC). However, a much higher incidence of reactivity was found with the lecithin free cardiolipin antigen cardchol (CC) which is an ethanolic solution containing 0.0175 per cent (w/v) cardiolipin and 0.3 per cent (w/v) cholesterol.

The incidence of reactivity and the titre of reactive sera were higher before than after heat inactivation at 56° C for 30 minutes with both antigens. However, the reactions with the usual cardiolipin antigen containing lecithin seemed more susceptible to heat inactivation than the reactions with cardchol (15)

apparent in undiluted and three fold diluted unheated serum i.e. in the first two tubes

6 It is concluded from these experiments that it has not been possible to demonstrate any serum factor that increases the complement fixing activity of the antilipoidal antibodies with cardiolipin antigen. The total effect of normal serum factors is a reduction in the reactivity of these antibodies as determined from the haemolysis curve. The effect of unheated normal serum is more pronounced than that of heat inactivated normal serum due among other things to the presence in the former of native complement.

7 The reduction in reactivity seen in most sera after heat inactivation cannot be explained as a result of changes in the influence of normal serum components. On the contrary heat inactivation seems to make normal serum factors less able to reduce the reactivity of syphilitic sera. It is probable, therefore that the reduction in reactivity after heat inactivation of syphilitic sera is due to a direct thermal effect on the antilipoidal immune globulins which may be more or less thermostable.

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The incidence of reactivity and the titre of reactive sera were higher before than after heat inactivation at 56° C for 30 minutes with both antigens However the reactions with the usual cardiolipin antigen containing lecithin seemed more susceptible to heat inactivation than the reactions with cardchol (15)

The varying results obtained with these two phospholipoid antigens might reflect differences between the immune globulins with which each antigen reacts

Experience with cardchol in syphilis serology rather supports such a hypothesis. With cardchol as antigen, sera from cases of primary syphilis gave titres equalling or even exceeding those obtained with the usual cardiolipin antigen containing lecithin. Sera from later stages of syphilis usually gave significantly lower titres with cardchol (8).

The fact that in TPI non-reactive lepromatous leprosy cardchol reacts with more sera and with higher titres than CLC antigen indicates that the complement-fixing antilipoidal immune globulins found in lepromatous leprosy without concomitant treponemal infection differ from those found in syphilis—at least from those found in the later stages of syphilis.

The heterogeneity of the antilipoidal immune globulins involved might, among others qualities, manifest itself as a difference in susceptibility to thermal influences.

On this background, it was considered of interest to study in more detail the influence of heat on the reactivity of leprosy sera with the two antigens, viz. CLC and cardchol.

## MATERIAL AND METHODS

**Serum.** The present study comprises serum samples from 32 male patients with lepromatous leprosy.

The blood samples were taken in Madras<sup>1</sup>. After separation of the clot the native serum samples were transported to Copenhagen by air in sealed glass ampoules without any preservative added and in unheated state at ambient temperature.

One serum from Osaka and one serum from Coonor both from patients suffering from lepromatous leprosy and both TPI non reactive were used for a supplementary study together with two sera from Copenhagen both from TPI reactive cases of secondary syphilis.

**Serology.** The serological examinations were performed in Copenhagen upon arrival of the sera.

All the sera were examined for content of treponemal antibody by means of the Treponema pallidum immobilization test (TPI) (9) and the fluorescent treponemal antibody test (FTA) (3).

All the specimens were also examined by means of two modifications of the complement fixation test: one with CLC antigen and the other with cardchol antigen. The technique has been described previously (11, 12). The titre is expressed as the log<sub>10</sub> value of the dilution which gives 50 per cent haemolysis calculated according to the Karber method (5). After examination in the unheated state each sample was divided into three aliquots which were heat inactivated at 56° C for 30 minutes, 60 minutes and 120 minutes respectively and then examined in the two complement fixation tests.

The four sera in the supplementary study were divided into eight aliquots each which were heat inactivated at 56° C for 5, 10, 20, 30, 45, 60 and 120 minutes respectively prior to examination in complement fixation tests with CLC and cardchol antigens.

<sup>1</sup> Thanks are due to Dr C. W. Chacko, Serologist, Madras Medical College for his kind assistance in obtaining the sera for this study and for providing the clinical data regarding the patients.

## RESULTS

A high incidence of anticomplementary activity in sera from cases of lepromatous leprosy has often been reported (1) 24 of the 32 sera (including both TPI reactive and TPI non reactive sera) showed some anticomplementary activity in the unheated state but none showed such activity after heat inactivation

TABLE 1

*Serological Examination of 32 Sera from Cases of Lepromatous Leprosy of Various Types and Varying Duration of Infection Results Given in log<sub>10</sub> Values*

| Sera No | Age years | Duration     |                  |     |         | CLC antigen Heat inactivation at 56°C |        |         | Cardchol antigen Heat inactivation at 56°C |        |         |
|---------|-----------|--------------|------------------|-----|---------|---------------------------------------|--------|---------|--|--------|---------|
|         |           | Leprosy type | Infect years     | TPI | FTA 200 | 30 min                                | 60 min | 120 min | 30 min                                     | 60 min | 120 min |
| 1       | 39        | L 1          | 5/12             | —   | ✓       | 0                                     | 0      | 0       | 1.026                                      | 0.978  | 0.716   |
| 2       | 24        | L 1          | 2                | —   | ✓       | 0                                     | 0      | 0       | 0.095                                      | 0      | 0       |
| 3       | 19        | L 2          | 1/12             | —   | ✓       | 0                                     | 0      | 0       | 0.716                                      | 0.334  | 0       |
| 4       | 25        | L 2          | 3 1 <sup>o</sup> | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 5       | 39        | L 2          | 6/12             | +   | R       | 1.050                                 | 0.835  | 0.716   | 0.811                                      | 0.740  | 0.620   |
| 6       | 25        | L 2          | 2                | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 7       | 22        | L 2          | 2                | —   | ✓       | 0.835                                 | 0      | 0       | 2.028                                      | 1.551  | 1.193   |
| 8       | 27        | L 2          | 3                | —   | ✓       | 0.143                                 | 0      | 0       | 0.286                                      | 0      | 0       |
| 9       | 30        | L 2          | 3                | +   | ✓       | 0                                     | 0      | 0       | 0.811                                      | 0.740  | 0.501   |
| 10      | 25        | L 2          | 5                | —   | ✓       | 0                                     | 0      | 0       | 0.095                                      | 0      | 0       |
| 11      | 45        | L 2          | 5                | +   | R       | 0                                     | 0      | 0       | 0.787                                      | 0.501  | 0.239   |
| 12      | 35        | L 2          | 6                | —   | ✓       | 0                                     | 0      | 0       | 1.241                                      | 1.097  | 0.573   |
| 13      | 50        | L 2          | 6                | +   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 14      | 50        | L 2          | 10               | +   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 15      | 23        | L 3          | 1/12             | —   | ✓       | 0                                     | 0      | 0       | 0.811                                      | 0.763  | 0.716   |
| 16      | 25        | L 3          | 6/12             | —   | ✓       | 0                                     | 0      | 0       | 0.191                                      | 0      | 0       |
| 17      | 45        | L 3          | 6/12             | —   | ✓       | 0                                     | 0      | 0       | 0.286                                      | 0      | 0       |
| 18      | 33        | L 3          | 1                | —   | ✓       | 0                                     | 0      | 0       | 0.620                                      | 0.334  | 0       |
| 19      | 24        | L 3          | 1                | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 20      | 35        | L 3          | 16 12            | —   | ✓       | 1.145                                 | 0      | 0       | 1.431                                      | 1.193  | 0.978   |
| 21      | 35        | L 3          | 3                | —   | ✓       | 0                                     | 0      | 0       | 1.002                                      | 0.620  | 0.429   |
| 22      | 28        | L 3          | 5                | —   | ✓       | 0                                     | 0      | 0       | 0.549                                      | 0.191  | 0       |
| 23      | 18        | L 3          | 8                | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 24      | 42        | L 3          | 9                | +   | R       | 1.026                                 | 0.620  | 0.334   | 0.740                                      | 0.620  | 0.620   |
| 25      | 35        | L 3          | 9                | —   | ✓       | 0                                     | 0      | 0       | 0.716                                      | 0.396  | 0.239   |
| 26      | 25        | L 3          | 10               | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 27      | 46        | L 3          | 12               | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 28      | 16        | L 3          | 10-16            | —   | ✓       | 0                                     | 0      | 0       | 2.147                                      | 2.098  | 1.765   |
| 29      | 77        | L 3          | 20               | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 30      | 32        | L 3          | 2 <sup>o</sup>   | —   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 31      | 40        | L ?          | 1 12             | +   | ✓       | 0                                     | 0      | 0       | 0  | 0      | 0       |
| 32      | 39        | L ?          | 9                | +   | R       | 1.050                                 | 0      | 0       | 0.835                                      | 0.501  | 0       |

CLC — ordinary leishman containing cardiolipin antigen

The results of serological examination of the heat inactivated sera are given in Table 1 16 out of 24 presumed non treponemal sera from cases of lepromatous leprosy showed reactivity with cardchol antigen after heat inactivation at 56° C for 30 minutes 11 of these were still reactive after inactivation for 60 minutes and 8 were reactive even after 120 minutes at 56° C



None of the 24 presumed non-treponemal sera reacted with CLC antigen after inactivation at 56° C for 60 minutes, despite the fact that two of these sera were strongly reactive with CLC antigen after normal heat inactivation at 56° C for 30 minutes. All reactions of these sera with CLC except one were weaker than the corresponding reactions with cardiolip

TABLE 2  
*Examination of 32 Lepromatous Leprosy Sera in TPI and FTA<sub>200</sub>*

|  | TPI         |     | FTA <sub>200</sub> |       |
|--|-------------|-----|--------------------|-------|
|  | No. of sera | %   | No. of sera        | %     |
| Reactive   | 8           | 25  | 4                  | 12.5  |
| Non reactive   | 24          | 75  | 28                 | 87.5  |
| Total  | 32          | 100 | 32                 | 100.0 |
| Total presumed non treponemal sera (TPI and FTA <sub>200</sub> non reactive) | 24 (75%)    |     |                    |       |
| Total presumed treponemal sera (TPI and/or FTA <sub>200</sub> reactive)      | 8 (25%)     |     |                    |       |

Table 2 summarizes the results of the treponemal tests. The incidence of TPI reactivity (25 per cent) is almost the same as that found in the previous study by *Schmidt* of leprosy sera from Madras (20 per cent) (14).

None of the patients had clinical lesions indicating treponemal disease. It was therefore considered justified to conclude that the 24 sera which did not react in the TPI or in the FTA<sub>200</sub> were non-treponemal. Table 3 shows the reports of previous studies concerning the reactivity in complement fixation test with cardiolipin antigen in TPI non-reactive sera from cases of lepromatous leprosy.

TABLE 3  
*Comparison of Results in the Present Study with Serological Data in Previous Studies of TPI Non Reactive Cases of Lepromatous Leprosy Examined in Complement Fixation Test with Cardiolipin (CLC) and Cardiolip (CC) Antigens*

|                        | Number of sera | Origin of sera | CLC reactive % | CLC reactive % |
|------------------------|----------------|----------------|----------------|----------------|
| Edmundson et al (1954) | 182            | Louisiana      | 64             |                |
| Koorj et al (1957)     | ?              | South Africa   | 17             |                |
| Rosetti et al (1958)   | 51             | Syria          | 14             |                |
| Fromm et al (1959)     | 289            | Philippines    | 4              |                |
| Schmidt (1959)         | 96             | Egypt          | 13             | 62             |
| Schmidt (1961)         | 88             | Madras         | 20             | 53             |
| Holst (1964)           | 24             | Madras         | 13             | 67             |

The results obtained in the present study, viz the finding that less than 20 per cent of these sera were reactive in complement fixation test with ordinary cardiolipin antigen, are in good agreement with previous reports. The high incidence of reactivity reported by *Edmundson et al* (4) with this antigen has not been supported by later investigations.

TABLE 4

*Effect of Heat Inactivation at 56° C for 30 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin*  
*24 TPI Non Reactive Sera from Cases of Lepromatous Leprosy*

| 56° C<br>inactivation | CF<br>result | CLC antigen |     | CC antigen |    |
|-----------------------|--------------|-------------|-----|------------|----|
|                       |              | No of sera  | %   | No of sera | %  |
| 30 min                | +            | 3           | 13  | 16         | 67 |
|                       | —            | 21          | 87  | 8          | 33 |
| 60 min                | +            | 0           | 0   | 11         | 46 |
|                       | —            | 24          | 100 | 13         | 54 |
| 120 min               | +            | 0           | 0   | 8          | 33 |
|                       | —            | 24          | 100 | 16         | 67 |

The high incidence of reactivity with cardiolipin antigen previously demonstrated by *Schmidt* (13, 14) with TPI non-reactive lepromatous leprosy sera from Egypt and India was confirmed in the present study

The effect of prolonged heat inactivation can be seen from Tables 4 and 5 from the graphs in Figs 1 and 2

The average loss in reactivity during the first half hour of heat inactivation at 56° C has been shown previously (15) to be about 0.5 log<sub>10</sub> value for presumed non-treponemal sera from cases of lepromatous leprosy

The present study shows that prolonged heat inactivation at 56° C

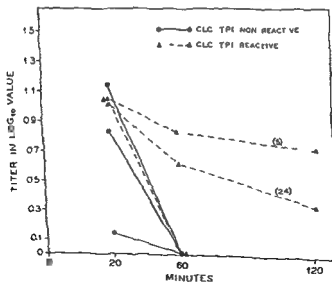


Fig 1

Variation in CLC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

None of the 24 presumed non-treponemal sera reacted with CLC antigen after inactivation at 56° C for 60 minutes, despite the fact that two of these sera were strongly reactive with CLC antigen after normal heat inactivation at 56° C for 30 minutes. All reactions of these sera with CLC except one were weaker than the corresponding reactions with cardchol

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|  | TPI         |     | FTA <sub>200</sub> |          |
|--|-------------|-----|--------------------|----------|
|  | No. of sera | %   | No. of sera        | %        |
| Reactive   | 8           | 25  | 4                  | 12.5     |
| Non reactive   | 24          | 75  | 28                 | 87.5     |
| Total  | 32          | 100 | 32                 | 100.0    |
| Total presumed non-treponemal sera (TPI and FTA <sub>200</sub> non reactive) |             |     |                    | 24 (75%) |
| Total presumed treponemal sera (TPI and/or FTA <sub>200</sub> reactive)      |             |     |                    | 8 (25%)  |

Table 2 summarizes the results of the treponemal tests. The incidence of TPI reactivity (25 per cent) is almost the same as that found in the previous study by *Schmidt* of leprosy sera from Madras (20 per cent) (14).

None of the patients had clinical lesions indicating treponemal disease. It was therefore considered justified to conclude that the 24 sera which did not react in the TPI or in the FTA<sub>200</sub> were non-treponemal. Table 3 shows the reports of previous studies concerning the reactivity in complement fixation test with cardiolipin antigen in TPI non-reactive sera from cases of lepromatous leprosy.

TABLE 3  
*Comparison of Results in the Present Study with Serological Data in Previous Studies of TPI Non Reactive Cases of Lepromatous Leprosy Examined in Complement Fixation Test with Cardiolipin (CLC) and Cardchol (CC) Antigens*

|                        | Number of sera | Origin of sera | CLC reactive % | CLC reactive % |
|------------------------|----------------|----------------|----------------|----------------|
| Edmundson et al (1954) | 182            | Louisiana      | 64             |                |
| Kooij et al (1957)     | 7              | South Africa   | 17             |                |
| Rosetti et al (1958)   | 51             | Spain          | 14             |                |
| Fromm et al (1959)     | 289            | Philippines    | 4              |                |
| Schmidt (1959)         | 96             | Egypt          | 13             | 62             |
| Schmidt (1961)         | 88             | Madras         | 20             | 53             |
| Holst (1964)           | 24             | Madras         | 13             | 67             |

The results obtained in the present study, viz the finding that less than 20 per cent of these sera were reactive in complement fixation test with ordinary cardiolipin antigen, are in good agreement with previous reports. The high incidence of reactivity reported by *Edmundson et al* (4) with this antigen has not been supported by later investigations.

TABLE 4

*Effect of Heat Inactivation at 56° C for 30, 60, and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin*  
*24 TPI Non Reactive Sera from Cases of Lepromatous Leprosy*

| 56° C<br>inactivation | CF<br>result | CLG antigen |     | CC antigen  |    |
|-----------------------|--------------|-------------|-----|-------------|----|
|                       |              | No. of sera | %   | No. of sera | %  |
| 30 min                | +            | 3           | 13  | 16          | 67 |
|                       | —            | 21          | 87  | 8           | 33 |
| 60 min                | +            | 0           | 0   | 11          | 46 |
|                       | —            | 24          | 100 | 13          | 54 |
| 120 min               | +            | 0           | 0   | 8           | 33 |
|                       | —            | 24          | 100 | 16          | 67 |

The high incidence of reactivity with cardiolipin antigen previously demonstrated by *Schmidt* (13, 14) with TPI non-reactive lepromatous leprosy sera from Egypt and India was confirmed in the present study.

The effect of prolonged heat inactivation can be seen from Tables 4 and 5 from the graphs in Figs 1 and 2

The average loss in reactivity during the first half-hour of heat inactivation at 56° C has been shown previously (15) to be about 0.5 log<sub>10</sub> value for presumed non treponemal sera from cases of lepromatous leprosy

The present study shows that prolonged heat inactivation at 56° C

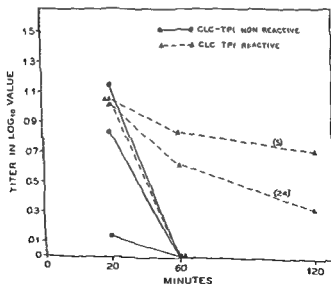


Fig 1

Variation in CLC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

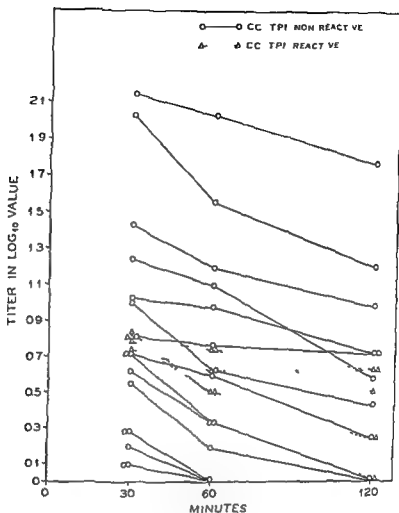


Fig. 9

Variation in CC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

TABLE 5

Effect of Heat Inactivation at 56° C for 30, 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardioli in Antigen with and without Lecithin 8 TPI Reactive Sera from Cases of Lepromatous Leprosy

| 56°C<br>inactivation | CF<br>result | CF antigen  |    | CL antigen  |    |
|----------------------|--------------|-------------|----|-------------|----|
|                      |              | No. of sera | %  | No. of sera | %  |
| 30 min               | +            | 3           | 38 | 3           | 61 |
|                      | -            | 1           | 62 | 3           | 38 |
| 60 min               | +            | 2           | 25 | 5           | 62 |
|                      | -            | 6           | 75 | 3           | 38 |
| 120 min              | +            | 2           | 25 | 4           | 50 |
|                      | -            | 6           | 75 | 4           | 50 |

TABLE 6

*Relation between Clinical Type of Lepromatous Leprosy and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Cases*

| Type of lepromatous leprosy | Number of sera | Results with cardchol after heat inactivation at 56°C |       |                              |          |      |                              |          |      |                              |
|-----------------------------|----------------|---|-------|------------------------------|----------|------|------------------------------|----------|------|------------------------------|
|                             |                | 30 min  |       |                              | 60 min   |      |                              | 120 min  |      |                              |
|                             |                | Reactive  |       | Mean log <sub>10</sub> value | Reactive |      | Mean log <sub>10</sub> value | Reactive |      | Mean log <sub>10</sub> value |
|                             |                | No  | %     |                              | No       | %    |                              | No       | %    |                              |
| L 1                         | 2              | 2   | (100) | 0.561                        | 1        | (50) | 0.489                        | 1        | (50) | 0.358                        |
| L 2                         | 7              | 3   | 71    | 0.624                        | 3        | 43   | 0.426                        | 2        | 29   | 0.224                        |
| L 3                         | 15             | 9   | 60    | 0.517                        | 7        | 47   | 0.382                        | 5        | 33   | 0.275                        |
| Total                       | 24             | 16  | 67    | 0.552                        | 11       | 46   | 0.403                        | 8        | 33   | 0.275                        |

TABLE 7

*Relation between Duration of Leprosy Infection Symptoms and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Sera from Patients with Lepromatous Leprosy*

| Duration of symptoms (years) | Number of sera | Results with cardchol after heat inactivation at 56°C |    |                              |          |    |                              |          |    |                              |
|------------------------------|----------------|---|----|------------------------------|----------|----|------------------------------|----------|----|------------------------------|
|                              |                | 30 min  |    |                              | 60 min   |    |                              | 120 min  |    |                              |
|                              |                | Reactive  |    | Mean log <sub>10</sub> value | Reactive |    | Mean log <sub>10</sub> value | Reactive |    | Mean log <sub>10</sub> value |
|                              |                | No  | %  |                              | No       | %  |                              | No       | %  |                              |
| 0.0-1.0                      | 8              | 6   | 75 | 0.458                        | 4        | 50 | 0.301                        | 2        | 25 | 0.179                        |
| 1.1-5.0                      | 8              | 7   | 88 | 0.686                        | 4        | 50 | 0.444                        | 3        | 38 | 0.325                        |
| 5.1-22.0                     | 8              | 3   | 38 | 0.513                        | 3        | 38 | 0.465                        | 3        | 38 | 0.322                        |
| Total                        | 24             | 16  | 67 | 0.552                        | 11       | 46 | 0.403                        | 8        | 33 | 0.275                        |

TABLE 8

*Detailed Study of Effect of Duration of Heat Inactivation at 56°C on the Reactivity with CLC and CLC Antigens of Two Lepromatous and Two Syphilitic Sera*

|               | Lepromatous leprosy Osaka 3 log <sub>10</sub> values |       | Lepromatous leprosy Connor 4 log <sub>10</sub> values |       | Secondary syphilis St. RA 46 log <sub>10</sub> values |       | Secondary syphilis St. RA 51 log <sub>10</sub> values |       |
|---------------|--|-------|---|-------|---|-------|---|-------|
|               | CLC  | LC    | CLC   | CC    | CLC   | CC    | CLC   | CC    |
| Unheated      | 1.575  | 2.052 | ac  | ac    | 1.384   | 1.288 | 1.193   | 1.288 |
| 56°C. 2 min   | 1.193  | 2.338 | 0.859   | 1.527 | 1.384   | 1.145 | 1.264   | 1.145 |
| 56°C. 5 min   | 1.097  | 1.926 | 0.239   | 1.527 | 1.336   | 1.145 | 1.431   | 1.145 |
| 56°C. 10 min  | 0  | 2.001 | 0   | 1.455 | 1.288   | 1.169 | 1.312   | 1.169 |
| 56°C. 20 min  | 0  | 1.885 | 0   | 1.312 | 1.431   | 1.193 | 1.217   | 1.217 |
| 56°C. 30 min  | 0  | 1.718 | 0   | 1.193 | 1.288   | 1.145 | 1.241   | 1.193 |
| 56°C. 45 min  | 0  | 1.394 | 0   | 1.097 | 1.288   | 1.002 | 1.193   | 1.050 |
| 56°C. 60 min  | 0  | 1.241 | 0   | 0.787 | 1.288   | 1.002 | 1.193   | 1.169 |
| 56°C. 120 min | 0  | 0.954 | ac  | ac    | 1.288   | 0.954 | 1.145   | 1.050 |

ac — anticomplementary

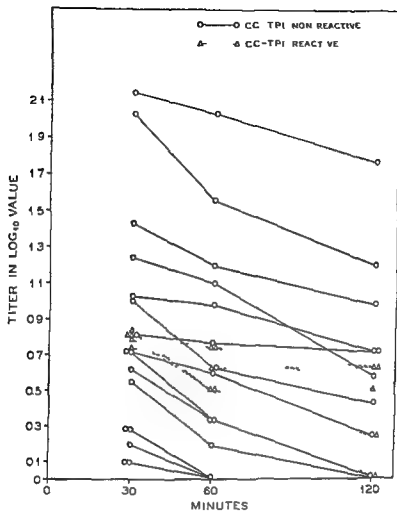


Fig 2

Variation in CC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting after inactivation for 30 minutes shown)

TABLE 5

Effect of Heat Inactivation at 56° C for 30 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin & TPI Reactive Sera from Cases of Lepromatous Leprosy

| 56° C<br>inactivation | CF<br>result | CF antigen |    | CL antigen |    |
|-----------------------|--------------|------------|----|------------|----|
|                       |              | No of sera | %  | No of sera | %  |
| 30 min                | +            | 3          | 38 | 3          | 62 |
|                       | —            | 5          | 62 | 3          | 38 |
| 60 min                | +            | 2          | 25 | 2          | 62 |
|                       | —            | 6          | 75 | 3          | 38 |
| 120 min               | +            | 2          | 25 | 4          | 50 |
|                       | —            | 6          | 75 | 4          | 50 |

TABLE 6

*Relation between Clinical Type of Lepromatous Leprosy and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Cases*

| Type of lepromatous leprosy | Number of sera | Results with cardchol after heat inactivation at 56°C |       |                              |          |      |                              |          |      |                              |
|-----------------------------|----------------|---|-------|------------------------------|----------|------|------------------------------|----------|------|------------------------------|
|                             |                | 30 min  |       |                              | 60 min   |      |                              | 120 min  |      |                              |
|                             |                | Reactive  |       | Mean log <sub>10</sub> value | Reactive |      | Mean log <sub>10</sub> value | Reactive |      | Mean log <sub>10</sub> value |
|                             |                | No  | %     |                              | No       | %    |                              | No       | %    |                              |
| L 1                         | 2              | 2   | (100) | 0.561                        | 1        | (50) | 0.489                        | 1        | (50) | 0.358                        |
| L 2                         | 7              | 11  | 71    | 0.624                        | 3        | 43   | 0.426                        | 11       | 29   | 0.224                        |
| L 3                         | 15             | 9   | 60    | 0.517                        | 7        | 47   | 0.382                        | 5        | 33   | 0.275                        |
| Total                       | 24             | 16  | 67    | 0.552                        | 11       | 46   | 0.403                        | 8        | 33   | 0.275                        |

TABLE 7

*Relation between Duration of Leprosy Infection Symptoms and Reactivity with Cardchol Antigen in 24 Presumed Non Treponemal Sera from Patients with Lepromatous Leprosy*

| Duration of symptoms (years) | Number of sera | Results with cardchol after heat inactivation at 56°C |    |                              |          |    |                              |          |    |                              |
|------------------------------|----------------|---|----|------------------------------|----------|----|------------------------------|----------|----|------------------------------|
|                              |                | 30 min  |    |                              | 60 min   |    |                              | 120 min  |    |                              |
|                              |                | Reactive  |    | Mean log <sub>10</sub> value | Reactive |    | Mean log <sub>10</sub> value | Reactive |    | Mean log <sub>10</sub> value |
|                              |                | No  | %  |                              | No       | %  |                              | No       | %  |                              |
| 0.0-1.0                      | 8              | 6   | 75 | 0.436                        | 4        | 50 | 0.101                        | 2        | 25 | 0.179                        |
| 1.1-5.0                      | 8              | 7   | 88 | 0.636                        | 4        | 50 | 0.411                        | 3        | 38 | 0.325                        |
| 5.1-22.0                     | 8              | 3   | 38 | 0.513                        | 3        | 38 | 0.465                        | 3        | 38 | 0.322                        |
| Total                        | 24             | 16  | 67 | 0.552                        | 11       | 46 | 0.403                        | 8        | 33 | 0.275                        |

TABLE 8

*Detailed Study of Effect of Duration of Heat Inactivation at 56°C on the Reactivity with CC and CLC Antigens of Two Lepromatous and Two Syphilitic Sera*

|              | Lepromatous leprosy (Sera 3)<br>log <sub>10</sub> values |       | Lepromatous leprosy (Sera 4)<br>log <sub>10</sub> values |       | Secondary syphilis (Sera 6)<br>log <sub>10</sub> values |       | Secondary syphilis (Sera 5)<br>log <sub>10</sub> values |       |
|--------------|--|-------|--|-------|---|-------|---|-------|
|              | CLC  | CC    | CLC  | CC    | CLC   | CC    | CLC   | CC    |
| Unheated     | 1.575  | 2.052 | ac   | ac    | 1.384   | 1.288 | 1.193   | 1.288 |
| 56°C/2 min   | 1.193  | 2.338 | 0.859  | 1.527 | 1.381   | 1.145 | 1.264   | 1.145 |
| 56°C/5 min   | 1.097  | 1.926 | 0.239  | 1.527 | 1.336   | 1.145 | 1.431   | 1.145 |
| 56°C/10 min  | 0  | 2.004 | 0  | 1.455 | 1.285   | 1.169 | 1.312   | 1.169 |
| 56°C/20 min  | 0  | 1.885 | 0  | 1.312 | 1.431   | 1.193 | 1.217   | 1.217 |
| 56°C/30 min  | 0  | 1.718 | 0  | 1.193 | 1.288   | 1.145 | 1.241   | 1.193 |
| 56°C/45 min  | 0  | 1.384 | 0  | 1.097 | 1.288   | 1.002 | 1.193   | 1.050 |
| 56°C/60 min  | 0  | 1.241 | 0  | 0.781 | 1.288   | 1.002 | 1.193   | 1.169 |
| 56°C/120 min | 0  | 0.954 | ac   | ac    | 1.288   | 0.954 | 1.145   | 1.050 |

ac = anticomplementary



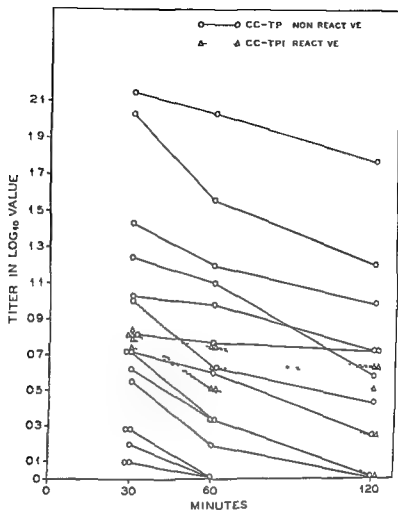


Fig 2

Variation in CC reactivity with the duration of heat inactivation at 56° C (only results for sera reacting, after inactivation for 30 minutes shown)

TABLE 5  
Effect of Heat Inactivation at 56° C for 30 60 and 120 Minutes on the Reactivity in Complement Fixation Test Using Cardiolipin Antigen with and without Lecithin & TPI Reactive Sera from Cases of Lepromatous Leprosy

| 56°C<br>inactivation | CF<br>result | CL antigen |    | CL antigen |    |
|----------------------|--------------|------------|----|------------|----|
|                      |              | No of sera | %  | No of sera | %  |
| 30 min               | +            | 3          | 38 | 3          | 62 |
|                      | —            | 5          | 62 | 3          | 38 |
| 60 min               | +            | 2          | 25 | 5          | 62 |
|                      | —            | 6          | 75 | 3          | 38 |
| 120 min              | +            | 2          | 25 | 4          | 50 |
|                      | —            | 6          | 75 | 4          | 50 |

and the type lepromatous lesion (L 1 L 2, L 3) (Table 6) nor does the heat stability of the reactions vary with the type of lepromatous leprosy.

Table 7 indicates that the percentage of reactive sera is lower in patients with long standing infections. However the reactivity in these sera seems to be influenced less by heat than the reactivity found in more recent cases i.e. of less than 5 years' duration. The small number of sera in each group does not allow any final conclusions on this point.

Table 8 gives the results of the supplementary study comparing the effect of heat inactivation of varying duration on the reactivity of two lepromatous and two syphilitic sera. Fig. 3 shows the variation in reactivity with the two antigens as a function of the duration of heat inactivation. It is evident that the reactions of the two syphilitic sera from Copenhagen are more heat stable than those of the two lepromatous sera from Osaka and Connor. This is especially the case for the reactions with CLC antigen.

### CONCLUSION

Based on the assumption that the reactivity in complement fixation tests with CLC and cardchol antigens reflects the thermal stability of the immune globulins involved, two conclusions can be drawn from these experiments:

1) Cardchol antigen in lepromatous leprosy reacts with a relatively heat stable immune globulin with which the CLC antigen does not react (at the same time cardchol probably reacts with the heat labile immune globulin with which CLC antigen reacts).

2) Both the relatively heat stable and the heat labile antipoidal immune globulin thus demonstrated in lepromatous leprosy differ in heat stability from the antipoidal immune globulins demonstrated in the two sera from cases of secondary syphilis.

### SUMMARY

32 sera from cases of lepromatous leprosy were examined in complement fixation test with lecithin free cardiolipin antigen (cardchol CC) as well as with standard cardiolipin antigen containing lecithin (LC) after heat inactivation at 56° C for 30, 60 and 120 minutes respectively.

On the basis of clinical examination and the results of the TPI 24 of these sera could be considered as originating from patients without concomitant treponemal infection.

16 of the 24 TPI non reactive sera (67 per cent) were reactive with cardchol antigen but only three (13 per cent) reacted with CLC antigen after 30 minutes inactivation.

Prolonged heat inactivation resulted in complete loss of reactivity with CLC antigen but 11 sera still showed reactivity with cardchol

results in a further decrease in reactivity during the second half hour. This decrease is more pronounced for CLC antigen ( $0.7 \log_{10}$  value) and less pronounced for cardchol antigen ( $0.2 \log_{10}$  value).

Of the eight TPI-reactive sera, two showed an entirely different pattern (Nos. 5 and 24), both reacting more strongly with CLC antigen than with cardchol antigen, and both reacting with CLC antigen after prolonged heat inactivation as well as with cardchol antigen. Three TPI-reactive sera (Nos. 9, 11 and 32), showed the same pattern of reactivity and heat stability in their reactions with the two antigens as that found with the presumed non-treponemal sera in the present study. The remaining three sera in the TPI-reactive group (Nos. 13, 14 and 31) were non-reactive with the two antigens in the complement fixation test.

There is no apparent relation between the reactivity with cardchol

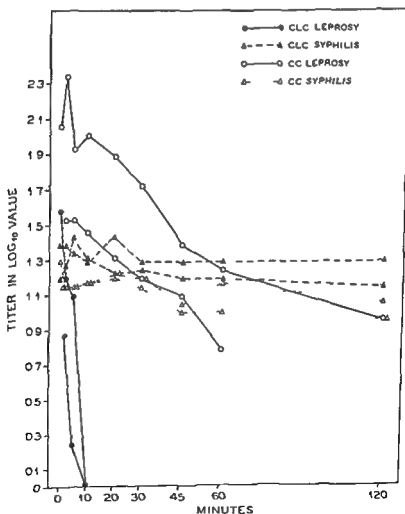


Fig. 3

Variation in CLC and CC reactivity with duration of heat inactivation at  $56^{\circ}\text{C}$  for two lepromatous leprosy sera and two syphilitic sera

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Department of Microbiology,  
and Broegmann's Research Laboratory for Microbiology, Bergen, Norway

## STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

### 2 Antigenic Formulas before and after Lysogenization

By

TOR HOFSTAD

Received 9 July 64

During a previous study of the agglutinogens in *Staphylococcus aureus* strains within the 80/81 complex (6), our type strains, and in particular the epidemic strain 263, went through repeated subcultures. The antigenic formulas and the phage patterns of strain 263 and other type strains both remained unchanged. The phenomenon of "antigenic loss variation" (14) was not encountered.

It has been shown in recent years (1, 3, 11, 12, 13) that the phage pattern of type 80 strains may be changed by lysogenization, i.e. by the introduction of a new phage into the strain by *in vitro* exposure of the strain to this phage, or presumably by contact with lysogenic, i.e. phage carrying, staphylococci in nature. Similar phage-induced conversion of some *Salmonella* strains has been followed by changes in somatic antigens (for references see (2)).

Therefore, before extending the studies of the antigenic structure of strain 263, it was found desirable to investigate in a controlled experiment whether or not the antigenic formula of this strain would change following lysogenization and repeated subculturing.

### MATERIALS AND METHODS

Strain 263 has been described earlier (6). In addition, some selected strains (11), shown in Table 2 were included in part of the study. These strains were kindly furnished by

and flooded on the surface of the plates were allowed to stand for 10 and 100 min from the

cells were inoculated. Single colonies of strains 80 and 81 and from the lysate area were typed. No secondary standard method. (15) The phages

antigen after 60 minutes' inactivation, and 8 of these showed reactivity with cardiol antigen even after 120 minutes' inactivation at 56° C

A supplementary experiment showed that the antilipoidal immune globulins found in secondary syphilis differ in heat stability from those found in lepromatous leprosy if it is assumed that the heat stability of the reactions with the two antigens in complement fixation tests reflects the heat stability of the immune globulins themselves

Two of the eight TPI-reactive sera from cases of lepromatous leprosy show a heat stability of complement fixation reactions rather similar to that found for secondary syphilis. Three other sera in this group behaved like the sera in the TPI non-reactive group, while the remaining three TPI-reactive sera were non-reactive with lipoidal antigens

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## STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF *STAPHYLOCOCCUS AUREUS*

### 2 Antigenic Formulas before and after Lysogenization

By

TOR HØFSTAD

Received 9 iv 64

During a previous study of the agglutinogens in *Staphylococcus aureus* strains within the 80/81 complex (6), our type strains, and in particular the epidemic strain 263, went through repeated subcultures. The antigenic formulas and the phage patterns of strain 263 and other type strains both remained unchanged. The phenomenon of "antigenic loss variation" (14) was not encountered.

It has been shown in recent years (1, 3, 11, 12, 13) that the phage pattern of type 80 strains may be changed by lysogenization, i.e. by the introduction of a new phage into the strain by *in vitro* exposure of the strain to this phage, or presumably by contact with lysogenic, i.e. phage carrying, staphylococci in nature. Similar phage-induced conversion of some *Salmonella* strains has been followed by changes in somatic antigens (for references see (2)).

Therefore, before extending the studies of the antigenic structure of strain 263, it was found desirable to investigate in a controlled experiment whether or not the antigenic formula of this strain would change following lysogenization and repeated subculturing.

### MATERIALS AND METHODS

Strain 263 has been described earlier (6). In addition some selected strains (11), shown in Table 2, were included in part of the study.

*Immunization of rabbits* agglutinin absorption and agglutination were carried out as described by Ordning (9) The techniques for preparation of the factor sera used were the same as in (6)

## RESULTS

The phage patterns and antigenic formulas of six variant strains obtained by lysogenization of strain 263 are shown in Table 1 The strains are designated according to the phages used for lysogenization, for example 263-KS6-3 means variant number three obtained by lysogenization with the phage KS6

TABLE 1  
Variant Strains Obtained by Lysogenization of Strain 263

| Strain    | Phage pattern | Antigenic formula       |
|-----------|---------------|-------------------------|
| 263       | 80/81/82/KS6  | $a_s(k_2)m$ 263 1 263 2 |
| 263 80 1  | 80/81/82/KS6  |                         |
| 263 81-1  | 80            |                         |
| 263 81-2  | 52/52A/80/KS6 |                         |
| 263 KS6 3 | 52/52A/80 KS6 |                         |
| 263 KS6-4 | 52/52A/81/82  |                         |
| 263-KS6 6 | 52/52A/80/82  |                         |

( $k_2$ ) - very weak

Other variant strains were isolated from the secondary growth in the lysis area of phage KS6, but they had all the typing pattern 52/52A/80/82 Strain 263-80-1 reacted weakly when typed with diluted phages

The variants were all typed with factor 262-1, 263-2,  $a_s$ ,  $h_1$ ,  $h_2$ ,  $k_1k_2$ ,  $k_1$ , and  $m$  sera The antigenic formulas were identical to that of the parent strain

The mother strain and the variants obtained were also examined for precipitinogens by double diffusion in agar While suspensions of the mother strain gave a weak, but distinct, polysaccharide A line (10), no such line was given by suspensions of the variant strains 263 KS6-3 and 263 KS6 6 This change, however, was only quantitative, as a weak polysaccharide A line could be obtained with concentrated extracts of the two variant strains (These examinations will be more fully dealt with in another paper)

The six variant strains were then subcultured daily on nutrient agar slopes Subcultures no 10, 20, 30, 40, and 50 were phage typed and examined in the above mentioned factor sera The antigenic formulas remained unchanged, and so also did the phage patterns with the exception of strain 263 81 2 When subculture no 50 had the same phage pattern as originally, subcultures no 10, 20, 30, and 40 were typed as 52/52A/80 82/KS6 (Routine Test Dilution (RTD) and  $RTD \times 1000$ )

Subculture no 50 of the six variant strains together with the parent strain were used for immunization of seven rabbits The seven strains exhausted each other's immune sera in cross-absorption experiments Finally, Rosenblum & Jackson's strains were typed with the same

factor sera as mentioned above (Table 2). With the exception of strains H101N (P60) and H101N(P53)(P60), all variants showed the same agglutinating patterns as the parent strains. Factor  $h_2$  serum could not be exhausted by nutrient agar or mannitol-salt agar cultures of the strains H101N, H101N(B Wm), H101N(P97) and H101N(P53) grown at 37° C.

TABLE 2

*Phage Patterns and Antigenic Formulas of Selected Staphylococcus aureus Strains<sup>1</sup> before and after Lysogenization*

| Strains         | Phage pattern        | Antigenic formula     |
|-----------------|----------------------|-----------------------|
| P184            | 80/81/82/hS6         | $a_3h_2$ 263 1 263 2  |
| P184(B Wm)      | 80/81/82/hS6         |                       |
| P184(P53)       | 52 52 4/80/81/82     |                       |
| P184(P97)       | 52 52 4/80/81/82     |                       |
| P184(P53)(B Wm) | 52/52 4/80/81/82     |                       |
| H101N           | 52/52 4/80/81/82 hS6 | $a_3$ 263 1 263-2     |
| H101N(B Wm)     | 52/52 4/80/81/82/hS6 |                       |
| H101N(P97)      | 52 52 4/80/81/82     |                       |
| H101N(P53)      | 52/52 4/80/81/82     |                       |
| H101N(P53)(P60) | 82                   | $a_3h_2$ 263 1 263 2  |
| H101N(P60)      | 81/82/hS6            |                       |
| P59             | 52 52 4/80/81/82/hS6 | $a_3h_2m$ 263 1 263 2 |
| P59(P60)        | 82/hS6               |                       |
| B Wm            | 52/52 4/80/81/82 hS6 | $a_3h_2$ 263 1 263 2  |
| B Wm(P60)       | 82/hS6               |                       |

<sup>1</sup> Parent strains and their phage variants from a study of Rosenblum & Jackson (11).

## DISCUSSION

While the phage pattern of strain 263 was readily changed by lysogenization, the antigenic formula was not. The alterations were, however, confined to gain or loss of sensitivity to phages within the 80/81 phage complex. This is in keeping with the clear correlation that was shown in the previous study (6) between the major 263-1 antigen and phage group 1 staphylococci, but not between the 263-1 antigen and other agglutinogens and lysins by particular type phages.

The stability of agglutinating pattern through subcultures is in accordance with studies by Grun & Kuhn (5) and Kretzschmar & Kretzschmar (8).

The gain of the heat-stable  $h$ -antigen in the variant strains H101N(P60) and H101N(P53)(P60) was unexpected. The parent strain H101N was therefore lysogenized once again in the same manner as before. This was most kindly brought about by Professor E. D. Rosenblum. The  $h$ -antigen could not be demonstrated in the variant strains H101N(P60) and H101N(P53)(P60) now obtained. Whether the presence of the  $h_2$  antigen in the original variants H101N(P60) and H101N



*Immunization of rabbits agglutinin absorption and agglutination* were carried out as described by Oeding (9) The techniques for preparation of the factor sera used were the same as in (6)

## RESULTS

The phage patterns and antigenic formulas of six variant strains obtained by lysogenization of strain 263 are shown in Table 1 The strains are designated according to the phages used for lysogenization, for example 263 KS6-3 means variant number three obtained by lysogenization with the phage KS6

TABLE 1  
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| Strain    | Phage pattern | Antigenic formula      |
|-----------|---------------|------------------------|
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| 263 80 1  | 80/81/82/KS6  |                        |
| 263 81-1  | 80            |                        |
| 263 81-2  | 52/52A/80/KS6 |                        |
| 263 KS6 3 | 52/52A/80/82  |                        |
| 263 KS6 4 | 52/52A/81/82  |                        |
| 263 KS6 6 | 52/52A/80/82  |                        |

(k) - very weak

Other variant strains were isolated from the secondary growth in the lysis area of phage KS6, but they had all the typing pattern 52/52A/80/82 Strain 263-80-1 reacted weakly when typed with diluted phages

The variants were all typed with factor 262-1, 263 2, a, h<sub>1</sub>, h<sub>2</sub>, k<sub>1</sub>k<sub>2</sub>, k<sub>3</sub>, and m sera The antigenic formulas were identical to that of the parent strain

The mother strain and the variants obtained were also examined for precipitinogens by double diffusion in agar While suspensions of the mother strain gave a weak, but distinct, polysaccharide A line (10), no such line was given by suspensions of the variant strains 263-KS6 3 and 263-KS6-6 This change, however, was only quantitative, as a weak polysaccharide A line could be obtained with concentrated extracts of the two variant strains (These examinations will be more fully dealt with in another paper)

The six variant strains were then subcultured daily on nutrient agar slopes Subcultures no 10, 20, 30, 40 and 50 were phage typed and examined in the above mentioned factor sera The antigenic formulas remained unchanged, and so also did the phage patterns with the exception of strain 263-81-2 When subculture no 50 had the same phage pattern as originally, subcultures no 10, 20, 30, and 40 were typed as 52/52A/80/82/KS6 (Routine Test Dilution (RTD) and RTD  $\times$  1 000)

Subculture no 50 of the six variant strains together with the parent strain were used for immunization of seven rabbits The seven strains exhausted each other's immune sera in cross-absorption experiments

Finally, *Rosenblum & Jackson's* strains were typed with the same

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## REDUCTION AND OXIDATION OF HYDROXYLAMINE BY EXTRACTS FROM *NEISSERIA MENINGITIDIS*

By

K. JYSSUM and P. E. JØNER

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Meningococci contain a TPN dependent glutamic dehydrogenase. An adaptation which is obviously necessary to obtain growth on a minimal medium with the ammonium ion as the only nitrogen source comprises an increased activity of a DPN dependent glutamic dehydrogenase (6, 7).

The literature dealing with the nitrate and nitrite reducing capacities of the Family *Neisseriaceae* has recently been reviewed by Berger (3). It seems to be generally agreed upon, that nitrate cannot be reduced by meningococci. On the other hand, Berger (3) was able to demonstrate a reduction of nitrile in four meningococcal strains. Two of these strains produced visible amounts of gas during the reaction. No significant quantities of ammonia were produced.

The present investigation was prompted by several observations concerning the effects of hydroxylamine in studies in which the energy metabolism of *Neisseria meningitidis* was the subject. As the work advanced, it became apparent that the effects were due to enzyme reactions, some of which had not previously been described from heterotrophs.

In the present communication two questions have been asked:

- 1) May meningococcal extracts catalyse the reduction of hydroxylamine?
- 2) May meningococcal extracts catalyse the oxidation of hydroxylamine?

Hydroxylamine reduction has been reviewed by Nason (15). The *Neurospora* hydroxylamine reductase was demonstrated to be a pyridine nucleotide specific metalloflavoprotein which catalysed the stoichiometric reduction of hydroxylamine to ammonia (22). The corresponding pyridine nucleotide-specific metalloprotein from soy bean leaves showed a specific requirement for  $Mn^{++}$  ions (18). Manganese ions have also been found to be a highly specific activator for the flavin stimulated adaptive pyridine nucleotide hydroxylamine reductase of *Azoto-*

(P53) (P60) was an expression of the phenomenon of 'antigenic loss variation', or simply due to contamination, can only be guessed

The results obtained in this small scale experiment strengthen the view that the agglutinating pattern of a *Staphylococcus aureus* strain is a much more stable property than phage susceptibility.

## SUMMARY

The antigenic formula of the *Staphylococcus aureus* strain 263 (phage type 80 81/82 KS6) remained unchanged after lysogenization and repeated subculturing. Similar results were obtained for some other staphylococcal strains within the 80 81 complex and their phage variants.

The results indicate that the agglutinating pattern in *Staphylococcus aureus* strains within the 80/81 complex is a stable and reliable property

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commercially from Sigma Chemical Co. Solutions of  $\text{NH}_2\text{OH}$  were prepared daily from  $\text{NH}_4\text{OH HCl}$  purchased from E. Merck, Darmstadt, Germany. The solutions were checked for the presence of nitrite before use since alkaline solutions are rapidly oxidized chemically.

## RESULTS

### *Reduction of Hydroxylamine*

Hydroxylamine reductase activity was assayed by the following procedure. The reaction mixture consisted of  $1.44 \times 10^{-4}$  M FMN 0.01 ml,  $2.4 \times 10^{-3}$  M TPNH 0.025 ml, 0.025 M G-6-P (sodium salt) 0.1 ml, 0.04 M  $\text{MnCl}_2$  0.01 ml,  $3 \times 10^{-4}$  M  $\text{NH}_2\text{OH}$  0.2 ml, meningococcus extract dilution 0.05 ml and 0.1 M Tris buffer pH 7.4 to a total volume of 1 ml.

The reaction was run at room temperature and stopped at the desired time by the addition of 0.5 ml of 13 per cent  $\text{I}_2$  in glacial acetic acid. The procedure of Roussos & Nason (18) was followed in order to determine the remaining hydroxylamine. In these experiments a zero time value was obtained by the addition of  $\text{I}_2$  plus glacial acetic acid to the mixture immediately after the reaction had been started. Endogenous and non enzymatic decrease in hydroxylamine content was measured in parallel assays in which enzyme and electron donor system were omitted singly.

From the results presented in Fig. 1 it is seen that hydroxylamine rapidly disappears in the system described. Since the meningococcal extracts used contain G-6-P dehydrogenase (8) the system described

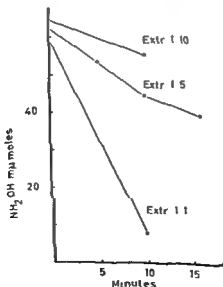


Fig. 1

Disappearance of hydroxylamine in the presence of TPNH, FAD and  $\text{Mg}^{++}$ .  
The effect of dilution of the meningococcus extract.

bacter (20) Mager (13) assumes that the TPNH specific hydroxylamine reductase and the TPNH specific sulphite reductase activities of *E. coli* may well be catalysed by the same enzyme. This author is of the opinion that the sulphite reduction represents the true physiological function of the enzyme.

The hydroxylamine reductases in many denitrifying bacteria do not seem to operate with DPNH or TPNH as electron donors. This is the case with *Micrococcus denitrificans* (11) as well as with *Pseudomonas denitrificans* (21). The major difference between the *Pseudomonas* reductase and that of *Micrococcus* appears to be the absence of FAD requirement from the latter.

Oxidation of hydroxylamine has so far only been reported from the chemolithotrophic bacterium *Nitrosomonas*. This area of nitrification has been reviewed by Aleem & Nason (2). Cell free preparations from *Nitrosomonas europaea* have been shown to oxidize hydroxylamine to nitrite when mammalian cytochrome C is used as the electron acceptor (5, 16). An unstable intermediate is apparently formed during the oxidation of hydroxylamine to nitrite. This may be NOH (4, 5). The experiments of Aleem *et al.* (1) were taken to suggest that nitrohydroxylamine is an intermediate in the oxidation of hydroxylamine, formed by the initial oxidative condensation of hydroxylamine and nitrite.

## MATERIALS AND METHODS

**Meningococcal strains.** The present study was performed with the virulent *Neisseria meningitidis* strain M6. The serologic nature of this strain has been reported previously (6). The strain was arbitrarily chosen as the main test microbe. The bacteria were maintained in the lyophilized state. When not otherwise stated the strain was adapted to growth on a minimal medium (6).

**Cell free extracts.** The methods used for growth, harvesting and extraction after ultrasonic disintegration of the cells have been described previously (10).

**Analytical procedures.** Hydroxylamine was determined according to the procedure described by Roussos & Nason (18) which involves the oxidation of hydroxylamine by iodine to nitrite. This procedure permits the determination of hydroxylamine in mmole quantities. Nitrite was measured by the method of Medina & Nicholas (14). Ammonia was determined by the Conway microdiffusion technique in conjunction with the phenylhypochlorite test for ammonia according to the procedure of Russell (19). Protein was analysed by the method of Johnson in the way it has previously been used (9).

**Equipment.** Spectrophotometry was performed either with a Hilger and Watts Uvispec spectrophotometer or in a Beckman DB spectrophotometer. In both instruments analyses were performed with 1 cm light paths.

**Cofactors and chemicals.** Mammalian cytochrome C (Sigma type III from horse heart) was oxidized by means of acidification with 1 N HCl to pH 3 and neutralization with an equivalent amount of alkali when the band at 550 m $\mu$  had vanished usually after 30 to 45 minutes at room temperature. Reduced cytochrome C was obtained by reduction with Pd/H<sub>2</sub>. A neutral cytochrome C solution mixed with approximately 0.2 g palladium asbestos (5 per cent) per 10 ml was first gassed with N<sub>2</sub> for 5 minutes and then with H<sub>2</sub> for 1 hour and again with N<sub>2</sub> for 5 minutes. The extent of reduction was tested by measuring the ratio of the absorbancy of the solution at 550 m $\mu$  to that at 565 m $\mu$ . The reduction of cytochrome C was essentially calculated according to the method of Nielsen & Lehniger (17) in terms of  $\frac{1}{2}$   $\Delta$  FeIII cytochrome C mmols (equal to mmols O). The pyridine nucleotide cofactors oxidized as well as reduced, the flavin cofactors and the G 6 P were also obtained.

commercially from Sigma Chemical Co. Solutions of  $\text{NH}_2\text{OH}$  were prepared daily from  $\text{NH}_2\text{OH}\cdot\text{HCl}$  purchased from E. Merck Darmstadt, Germany. The solutions were checked for the presence of nitrite before use since alkaline solutions are rapidly oxidized chemically.

## RESULTS

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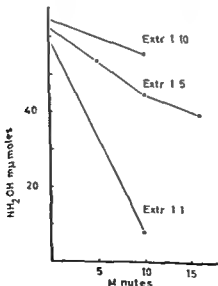


Fig. 1

Disappearance of hydroxylamine in the presence of TPNH, FAD and  $\text{Mg}^{++}$ .  
The effect of dilution of the meningococcus extract

was able to maintain almost all the added pyridine nucleotide in the reduced state in the presence of G-6-P as electron donor

The cofactor requirement of the system presented was analysed in the way it has been recorded in Table 1

TABLE 1  
*The Effect of Various Cofactors and Electron Donors on the Hydroxylamine Reduction by Extracts from Neisseria meningitidis*

| Experiment no | Factor omitted from the system        | NH OH at the start $\mu$ moles | NH OH recovered after 10 min $\mu$ moles |
|---------------|---------------------------------------|--------------------------------|--|
| I             | None                                  | 47.5                           | 20.0                                     |
|               | FMN                                   | 47.5                           | 34.0                                     |
|               | TPNH                                  | 47.5                           | 25.6                                     |
|               | G-6-P                                 | 47.5                           | 23.1                                     |
|               | Mn <sup>++</sup>                      | 47.5                           | 42.7                                     |
| II            | None                                  | 50.4                           | 21.8                                     |
|               | TPNH and G-6-P                        | 50.4                           | 43.1                                     |
|               | G-6-P and Mn <sup>++</sup>            | 50.4                           | 48.8                                     |
|               | TPNH and Mn <sup>++</sup>             | 50.4                           | 49.0                                     |
|               | FMN and G-6-P                         | 50.4                           | 35.5                                     |
|               | FMN and TPNH                          | 50.4                           | 29.6                                     |
|               | FMN and Mn <sup>++</sup>              | 50.4                           | 47.9                                     |
| III           | None                                  | 55.6                           | 21.3                                     |
|               | TPNH, G-6-P and Mn <sup>++</sup>      | 55.6                           | 55.0                                     |
|               | FMN, G-6-P and Mn <sup>++</sup>       | 55.6                           | 52.0                                     |
|               | FMN, TPNH and Mn <sup>++</sup>        | 55.6                           | 52.3                                     |
|               | FMN, TPNH and G-6-P                   | 55.6                           | 47.3                                     |
|               | FMN, TPNH, G-6-P and Mn <sup>++</sup> | 55.6                           | 55.6                                     |
|               | Extract                               | 55.6                           | 52.5                                     |

The experimental data show that Mn<sup>++</sup> is essential for the reaction. It is also seen that either TPNH or G-6-P must be added as a source of electrons. Also, there seems to be a significant increase in hydroxylamine disappearance when FMN is added to the system.

In following series of experiments it was found that DPNH could replace TPNH in the system described. There is some indication, however, that the latter is slightly more effective as electron donor in the meningococcal system.

The DPNH and TPNH oxidases of *Neisseria meningitidis* are very active (8, 9) and compete strongly for reduced pyridine nucleotide. The stimulatory effect of TPNH on the rates of hydroxylamine reduction is shown in Table 1. With a view to the interfering oxidase activities it may be understood why it was not possible to do stoichiometric or kinetic studies based on the rate of oxidation of the reduced pyridine nucleotides when these were used as the only electron donors.

The hydroxylamine reductase in crude extracts from *Neisseria men-*

*meningitidis* could be stored for at least 14 days at  $18^{\circ}\text{C}$  without appreciable loss of activity

The disappearance of hydroxylamine from the assay system was accompanied by ammonia formation. This was demonstrated in a reaction system similar to that previously described with the exception that a phosphate buffer was used instead of the Tris buffer. The procedure was essentially as that described by Zucker & Nason (22). Endogenous and non enzymatically formed ammonia was measured in parallel assays. A corresponding zero time control was also included. In such an experiment  $2\text{ }\mu\text{g NH}_3$  was recovered where a quantitative recovery of the hydroxylamine disappeared should have given  $3.4\text{ }\mu\text{g NH}_3$ .

### Oxidation of Hydroxylamine

Cell free preparations from *Neisseria meningitidis* actively catalyse the reduction of mammalian cytochrome C when hydroxylamine is used as the electron donor. This activity was first measured in a system with the following components: Oxidized cytochrome C  $0.06\text{--}0.07\text{ }\mu\text{moles}$ ,  $\text{NH}_2\text{OH}$   $10\text{ }\mu\text{moles}$ ,  $0.1\text{ M}$  Tris buffer pH 7.2 to a total volume of  $2\text{ ml}$ . The experiments were performed at room temperature and followed by the reading of absorbancy at  $550\text{ m}\mu$ . In Fig 2 the results from some experiments in this technique have been presented.

*Neisseria meningitidis* contains an active cytochrome C oxidase which also oxidizes reduced mammalian cytochrome C in the way it has been

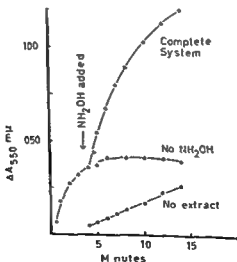


Fig 2

Reduction of mammalian cytochrome C by hydroxylamine in the presence of meningococcus extract  
Cytochrome C added at zero time hydroxylamine as shown



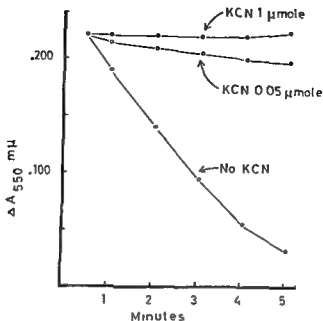


Fig 3

Oxidation of reduced mammalian cytochrome C by meningoecoccus extract  
Inhibition by cyanide

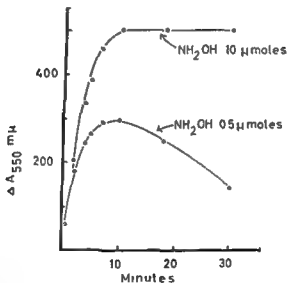


Fig 4

Reduction of mammalian cytochrome C by hydroxylamine in the presence  
of meningoecoccus extract Reoxidation by cytochrome oxidase

shown by the experiments recorded in Fig 3 This cytochrome oxidase effect becomes apparent in the assay of hydroxylamine oxidation When small quantities of substrate were used, prolonged incubation resulted in a reoxidation of the cytochrome This effect has been demonstrated in Fig 4 It is of course desirable to be able to study the rate of reduction of cytochrome without the competition from the oxidase Accord-

ingly, the effect of the conventional oxidase inhibitor cyanide on the experimental system was investigated. Unfortunately also the rate of reduction of cytochrome C which is mediated by hydroxylamine is to some extent inhibited by this chemical.

TABLE 2

*The Effect of Cyanide on the Reduction of Mammalian Cytochrome C by Hydroxylamine in the Presence of Extracts from Neisseria meningitidis*

|                        | Reduction of cytochrome C<br>± Fe <sup>III</sup> cytochrome C μmoles |            |
|------------------------|--|------------|
|                        | Cyanide<br>1 μmole   | No cyanide |
| NH-OH as the substrate | 9.7  | 13.0       |
| Endogenous metabolism  | 6.5  | 5.9        |
| Reduction due to NH-OH | 3.2  | 7.1        |

Reduction of cytochrome C was calculated after 5 minutes.  
Chemical reduction from NH-OH deducted.

From some data presented in Table 2 it is seen that cyanide in the concentration used inhibits the reaction under study approximately 50 per cent. Obviously, the endogenous reduction of cytochrome C is not similarly inhibited. When the oxidase is blocked, we find an increased endogenous reduction, presumably because the reoxidation is eliminated. With 1 μmole cyanide the hydroxylamine mediated cytochrome C reduction was 50 to 55 per cent inhibited. With 0.1 μmole cyanide, however, the reduction such as measured was no more than 5 per cent inhibited while the cytochrome oxidase was virtually stopped. The quantity 0.1 μmole has accordingly been chosen for the following experiments in which cyanide was deemed necessary.

Fluoride in a quantity of 10 μmoles did not interfere with the rate of reduction of cytochrome with hydroxylamine.

The reduction of cytochrome C by hydroxylamine is not dependent on P<sub>i</sub>. On the contrary, the addition of 3 μmoles P<sub>i</sub> resulted in approximately 20 per cent inhibition of the reduction rate when more - 1

n  
if a certain extract was incubated with hydroxylamine and cytochrome C under atmosphere of nitrogen a gas was formed. The composition of this gas has not been analysed.

The hydroxylamine mediated reduction of cytochrome C in meningo-coccal extracts is still present after dialysis for 22 hours against Tris buffer. Extracts may be kept at 18° C for more than 14 days without significant loss of activity.

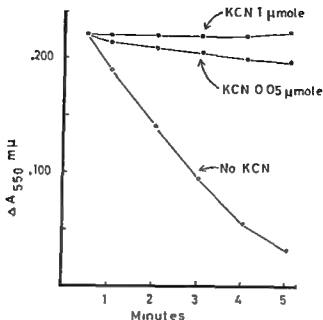


Fig 3

Oxidation of reduced mammalian cytochrome C by meningococcus extract  
Inhibition by cyanide

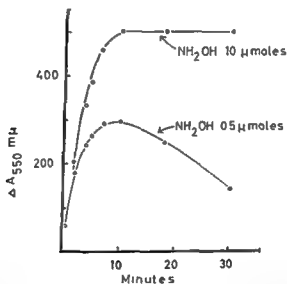


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has not previously been found in heterotrophs. The reaction has been discussed in relation to that found in *Nitrosomonas europaea*.

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## DISCUSSION

Hydroxylamine is removed from solutions in the presence of meningococcal extracts. This can be achieved in two ways. Either we may supply the extracts with excess of TPNH or DPNH as an electron source, or we may provide the system with excess of oxidized mammalian cytochrome C as an electron acceptor. Both reactions are at present taken to be enzyme catalysed reactions.

The first activity is assumed to be the result of a hydroxylamine reductase in the meningococcal extracts, corresponding to those described from other microbes. The enzyme is similar to that found in soy bean (22) since manganese ions serve as an activator and either FAD or FMN meets the flavin requirement. Like the enzymes from soy bean and *Neurospora* the reaction product appears to be ammonia. In *Neisseria meningitidis* the hydroxylamine reductase seems to be a constitutional enzyme.

The second activity which is considered in this paper may be assumed to signify a hydroxylamine oxidase. In any case, mammalian cytochrome C is reduced by hydroxylamine in the presence of cell free extracts from *Neisseria meningitidis*. The reaction product of this system has not been identified, but it is neither nitrite, nor nitrate. The product may turn out to be an unstable intermediate like NOH which is non-enzymatically oxidized to for instance  $N_2O$  (12).

The reaction found in *Neisseria meningitidis* seems in many respects to be similar to that found in *Nitrosomonas europaea* (5). In contrast to that system, however, it has not been possible to demonstrate a synthesis of nitrite in the meningococcal system, even in the absence of cyanide. The cytochrome C reduction is approximately 50 per cent inhibited by  $5 \times 10^{-4}$  M cyanide. This inhibition may be due to the formation of a chelate with a metal cofactor. Such a cofactor has not been identified. The quantity of cyanide necessary to inhibit the cytochrome oxidase of *Neisseria meningitidis* 100 per cent does not inhibit the cytochrome reduction from hydroxylamine more than approximately 5 per cent.

The hydroxylamine-cytochrome C reductase of *Neisseria meningitidis* seems to be a constitutional enzyme.

## SUMMARY

Meningococcal cell free extracts possess an activity corresponding to a hydroxylamine reductase. The reaction utilizes reduced pyridine nucleotides as electron donors and requires added flavin nucleotides for maximal activity.  $Mn^{++}$  is a specific activator. The product of the reaction appears to be ammonia.

Meningococcal cell free preparations also have been shown to reduce mammalian cytochrome C in the presence of hydroxylamine. The reaction is assumed to signify a hydroxylamine oxidase, an enzyme which



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## RESULTS

The reduction of mammalian cytochrome C which is catalysed by extracts from *Neisseria meningitidis* does not seem to need specific co factors. An inhibition by fairly high concentrations of cyanide however may indicate a metal cofactor requirement (6).

A study of phosphorylation calls for the addition of ADP and  $P_i$  to the mixture. Also the addition of  $Mg^{++}$  seems to be required by all known phosphorylations coupled to an electron flux to cytochrome C. Accordingly the influence of these factors on the hydroxylamine mediated reduction of cytochrome C in meningococcal extracts was investigated.

From some data presented in Table 1 it is seen that the addition of each of the three factors alone inhibits the degree of reduction when measured over a period of five minutes.

TABLE 1  
The Inhibition of Hydroxylamine Mediated Reduction of Cytochrome C by  $Mg$ ,  $P_i$  and ADP

| Substance added | Quantity         | Per cent activity |
|-----------------|------------------|-------------------|
| None            |                  | 100.0             |
| $P_i$           | 3.2 $\mu$ moles  | 78.7              |
| ADP             | 5.0 $\mu$ moles  | 14.5              |
| $Mg$            | 10.0 $\mu$ moles | 33.9              |
| $Mg$            | 5.0 $\mu$ moles  | 40.6              |

Activity was calculated in terms of  $\Delta A_{520 \text{ m}\mu}$  average per minute over an observation time of five minutes. Endogenous and chemical reduction deducted.

According to these data it appears natural to reduce the concentration of ADP and  $Mg$  as much as possible. On the other hand the quantities of these reagents should be chosen so as to limit the endogenous incorporation of  $P_i$ . Since a major part of this incorporation is due to the polynucleotide phosphorylase (5) the quantities selected should be unfavourable for this enzyme reaction. For the present studies 5  $\mu$ moles  $Mg$  were used giving a concentration of 2.5 mM. This is still far from the optimal concentration for the polynucleotide phosphorylase which is approximately 1 mM (5). The quantity of ADP was reduced to 2.5  $\mu$ moles an amount which when added alone still inhibits the reduction of cytochrome C. A total of 1.6  $\mu$ moles  $P_i$  was added to the system. This results in a ratio ADP to  $P_i$  of 1.56 which is still far from the ratio 0.78 found optimal for the polynucleotide phosphorylase (5).

When the mixture of  $Mg$ , ADP and  $P_i$  finally decided upon was added to the system the degree of reduction of cytochrome C was increased rather than the opposite. In Fig. 1 the time course curves from such an experiment are presented. When considering the linearity of the supplemented system during the first three to four minutes of observation as opposed to the curve representing the basal system it be



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Received 17 iv 64

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In the present paper one question has been asked: Is the reduction of mammalian cytochrome C by hydroxylamine in the presence of meningococcal extracts coupled with a phosphorylation of ADP to ATP?

The use of added ferricytochrome C as electron acceptor in studies of oxidative phosphorylation was briefly reported by Slater (11). The methods, however, were elaborated by Nielsen & Lehninger (8) and of Borgstrom, Sudduth & Lehninger (1).

## MATERIALS AND METHODS

The methodology and experimental manipulations employed in this investigation were analogous to those previously used (6). The test microbe was the meningococ

osphate uptake was determined. The method has been described by Nielsen & Lehninger (8). Measurements were carried out in cuvet cells as described (6). The calculational manipulations were based on Lehninger (8).

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## MATERIALS AND METHODS

The methodology and experimental manipulations employed in this investigation were analogous to those previously used (6). The test microbe was the meningococcus strain M6 maintained as previously described.

*Determination of orthophosphate uptake.* The phosphate uptake was determined

incorporation is due to the activity of the polynucleotide phosphorylase and the adenylate kinase (5). A heavy incorporation, however, may also be caused by other exchange reactions independent of electron transport. This type of exchange is strongly influenced by several conventional inhibitors (*Jyssum* & *Jyssum* unpubl. results). It is therefore clear that the endogenous uptake of inorganic phosphate must be studied in great detail in order to permit any definite conclusions to be drawn regarding a phosphorylation coupled to the electron flux.

In the present experiments the endogenous phosphorylation had to be considered under two different conditions. The first is concerned with cytochrome C when added alone. The other with hydroxylamine when present but without an added electron acceptor.

Perusal of the data presented in Table 2 shows that the addition of cytochrome C results in a pronounced endogenous reduction. This electron flow is accompanied by a small and variable uptake of inorganic phosphate. We will call this an "endogenous cytochrome phosphorylation".

TABLE 2  
*Reduction of Ferricytochrome C and Incorporation of  $P_i$  in the Presence of Meningococcal Extracts*  
*Analysis of the Endogenous Metabolism and of Hydroxylamine Effects*

| Expt. no.                |   |                        | 1    | 2    | 3    |
|--------------------------|---|------------------------|------|------|------|
| Endogenous Metabolism    | $P_i$ Uptake  | Extr + Cyto            | 23.2 | 25.4 | 40.5 |
|                          |   | Extr                   | 23.8 | 25.4 | 38.5 |
|                          |   | Effect of Cyto         | 1.4  | —    | 2.0  |
|                          | Red. of Cytochrome  |                        | 11.5 | 11.8 | 7.8  |
| Hydroxylamine Metabolism | $P_i$ Uptake No Electron Flux to Cytochrome                   | Extr + $NH_2OH$        | 24.3 | 26.4 | 42.1 |
|                          |   | Extr                   | 23.8 | 25.4 | 38.5 |
|                          |   | Effect of $NH_2OH$     | 0.5  | 1.0  | 3.6  |
|                          | $P_i$ Uptake Electron Flux to Cytochrome                      | Extr + Cyto + $NH_2OH$ | 30.8 | 28.8 | 46.2 |
|                          |   | Extr + Cyto            | 25.2 | 25.4 | 40.5 |
|                          |   | Effect of $NH_2OH$     | 5.6  | 3.4  | 5.7  |
|                          | $P_i$ Uptake due to $NH_2OH$ Mediated Reduction of Cytochrome |                        | 5.1  | 2.4  | 2.1  |
|                          | Red. of Cytochrome due to $NH_2OH$ *                          |                        | 4.2  | 5.5  | 2.9  |
|                          | P/O Ratio   |                        | 1.2  | 0.4  | 0.7  |

\* Hydroxylamine mediated reduction was calculated by subtracting the sum of endogenous reduction and chemical reduction.

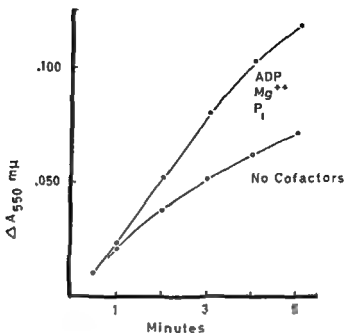


Fig 1

The influence of a combined addition of ADP,  $Mg^{++}$  and  $P_i$  on the reduction of cytochrome c by hydroxylamine in the presence of meningococcus extract

comes apparent that the effect of the factors added is not on the initial rate of the reaction, but on the faculty of the system to maintain a high initial rate of reduction over a considerable period of time. In other words, a rate limiting factor appearing very early in the basal system has been eliminated or rather significantly postponed as the result of a combined addition of  $Mg^{++}$ , ADP and  $P_i$ .

After the establishment of this experimental system studies concerning the incorporation of inorganic orthophosphate were started. The type of experiments recorded in Table 2 was performed in order to obtain a "balance sheet" concerning the incorporation of  $P_i$  during endogenous electron flow, in the presence of hydroxylamine without electron flow to cytochrome c, and during electron flow caused by hydroxylamine. Since we primarily were interested in the electron transport to cytochrome c cyanide was added in order to inhibit the cytochrome oxidase such as previously described (6).

The use of cyanide in such experiments is open to some discussion. Cyanide forms a complex with cytochrome c (3). It is assumed, however, that the rate of this reaction with the cyanide concentration chosen, is too slow to inhibit the rate of reduction of cytochrome c in the short intervals studied.

Previous investigations concerning the incorporation of  $^{32}P_i$  in the presence of extracts from *Neisseria meningitidis* have demonstrated considerable incorporation independent of electron flux. The radioactivity is located mainly in ADP and ATP under the present experimental and analytical conditions. A major part of this "endogenous"

incorporation is due to the activity of the polynucleotide phosphorylase and the adenylate kinase (5). A heavy incorporation however may also be caused by other exchange reactions independent of electron transport. This type of exchange is strongly influenced by several conventional inhibitors (*Jyssum* & *Jyssum* unpubl. results). It is therefore clear that the endogenous uptake of inorganic phosphate must be studied in great detail in order to permit any definite conclusions to be drawn regarding a phosphorylation coupled to the electron flux.

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|                          | Red. of Cytochrome   |                     | 11.3 | 11.8 | 7.8  |
| Hydroxylamine Metabolism | $P_i$ Uptake No Electron Flux to Cytochrome  | Extr + NH OH        | 24.3 | 26.4 | 42.1 |
|                          |  | Extr                | 23.8 | 25.4 | 38.5 |
|                          |  | Effect of NH OH     | 0.5  | 1.0  | 3.6  |
|                          | $P_i$ Uptake Electron Flux to Cytochrome   | Extr + Cyto + NH OH | 30.8 | 28.8 | 46.2 |
|                          |  | Extr + Cyto         | 25.2 | 25.4 | 40.5 |
|                          |  | Effect of NH OH     | 5.6  | 3.4  | 5.7  |
|                          | $P_i$ Uptake due to NH OH Mediated Reduction of Cytochrome   |                     | 5.1  | 2.4  | 2.1  |
|                          | Red. of Cytochrome due to NH OH  |                     | 4.3  | 5.5  | 2.9  |
|                          | $P/O$ Ratio  |                     | 1.2  | 0.4  | 0.7  |
|                          | Hydroxylamine mediated reduction as calculated by subtracting the sum of endogenous reduction and chemical reduction |                     |      |      |      |

The complete system contained ADP 0.1 mmoles Ferricytochrome C  
 Meningococcus extract dilution 0.2 ml  
 of 2 ml. tube

Since hydroxylamine, the substrate of the present study, is an inhibitor of several enzyme reactions in the concentration actually used an effect on the endogenous  $P_i$  incorporation has to be considered. From the data presented in Table 1 it is seen that the endogenous incorporation of  $P_i$  is in fact increased in the presence of hydroxylamine even when an electron acceptor is absent. This phosphorylation will be referred to as the "endogenous hydroxylamine phosphorylation".

The experiments presented in Table 2 may on the whole be taken as an indication that a phosphorylation indeed occurs concomitant with the hydroxylamine mediated reduction of cytochrome C.

TABLE 3  
Effects of Various Treatments and Chemical Agents on Phosphorylation Associated with Hydroxylamine Mediated Reduction of Cytochrome C in *Meningococci*

| Experiment no                      |   | 1   | 2   | 3  | 4      | 5   | 6  | 7        | 8      |
|------------------------------------|---|-----|-----|----|--------|-----|----|----------|--------|
| Treatment of Extract               |   | -   | -   |    | Ageing | -   |    | Dialysis | Ageing |
| Chemical Agent Added *             |   | -   | DNP | As | -      | F   | F  | -        | F      |
| Endogenous Metabolism<br>mmoles    | $P_i$ Uptake due to Cytochrome                              | 37  | 41  | 08 | 49     | -   | 09 | -        | -      |
|                                    | Reduction of Cytochrome                                     | 157 | 119 | 92 | 58     | 117 | 31 | 21       | 52     |
| Hydroxylamine Metabolism<br>mmoles | $P_i$ Uptake due to $NH_2OH$ No Electron Flux to Cytochrome | 38  | 45  | 09 | 75     | 20  | 78 | 69       | 28     |
|                                    | $P_i$ Uptake due to $NH_2OH$ 1 Electron Flux to Cytochrome  | 89  | 91  | 07 | 26     | 18  | 86 | 67       | 31     |
|                                    | $P_i$ Uptake due to $NH_2OH$ Mediated Red. of Cytochrome    | 51  | 46  |    |        |     | 08 | -        | 02     |
|                                    | Reduction of Cytochrome due to $NH_2OH$                     | 29  | 24  | 39 | 72     | 10  | 41 | 24       | 50     |
|                                    | P/O Ratio   | 17  | 19  |    |        |     | 02 |          |        |

\*  $2 \times 10^{-4} M$   
 $3 M$   
 $\times 10^{-4} M$

The experimental system and calculations were analogous to those described in the legend of Table 1.

Common to systems in which phosphorylations are coupled to electron transport "above" substrate level is the capacity to become uncoupled. Perhaps the least drastic among the classical uncoupling procedures is ageing. In Table 3 the effect of ageing has been investigated among some other uncoupling procedures.

Evidently the phosphorylation coupled to the reduction of cytochrome C from hydroxylamine is completely uncoupled. On the other hand, the reduction is increased rather than reduced. The latter observation is in agreement with previous findings concerning the stability of the electron transport system in question (6).

A comparison of the coupled system with the uncoupled one permits conclusions to be drawn regarding the nature of the two kinds of endogenous phosphorylation previously referred to.

The endogenous cytochrome phosphorylation may on the one hand be considered a true electron transport phosphorylation. On the other hand, it may be the result of an increased endogenous phosphorylation on the substrate level due to a reestablishment of the electron flow. From the data presented in Table 3 it is seen that the endogenous cytochrome phosphorylation is not uncoupled after ageing of the extract. The endogenous effect of cytochrome C is accordingly not interpreted as an electron transport phosphorylation.

The endogenous hydroxylamine phosphorylation may be an effect independent of electron flux. Another explanation would be that it is due to an electron flux which is independent of added cytochrome C. Such an electron flow might be due to an endogenous pool of oxidized cytochrome C, or electrons might somehow escape the cyanide block established. Studies with aged extracts clearly demonstrate that the endogenous hydroxylamine phosphorylation is unimpaired by this treatment in the way it has been illustrated in Table 3. The endogenous hydroxylamine phosphorylation is accordingly construed as an effect unrelated to electron flux phosphorylation.

In successive series of experiments some conventional inhibitors and uncoupling agents were tested. In the experiments recorded in Table 3 one observation has been made which is in disagreement with the general experience from electron transport phosphorylation in bacteria.

The phosphorylation is obviously strongly but completely inhibited by the addition of 10  $\mu$ moles fluoride without any significant effect on the reduction of cytochrome C. Fluoride in this concentration inhibits the endogenous cytochrome phosphorylation without a similar effect on the endogenous hydroxylamine phosphorylation. Obviously, the mechanisms involved in the two endogenous effects are entirely different. Arsenate which also uncouples the phosphorylation due to a reduction of cytochrome C by hydroxylamine, seems to reduce the endogenous cytochrome phosphorylation as well as the endogenous hydroxylamine phosphorylation.



## DISCUSSION

A reduction of ferri cytochrome C is mediated by hydroxylamine in the presence of extracts from *Neisseria meningitidis*. When the experimental system is supplemented with  $Mg^{++}$ , ADP and labelled orthophosphate the reduction is accompanied by a phosphorylation.

In the experiments performed in order to demonstrate this effect the reoxidation of ferrocytochrome C was prevented by an inhibition of the cytochrome oxidase with cyanide. With the cyanide concentration chosen the reduction of cytochrome by hydroxylamine was approximately 5 per cent inhibited (6).

The experimental system which was used in the present investigation was arranged in order to obtain maximal reduction of cytochrome C with a minimum of endogenous uptake of radioactive orthophosphate. No special effort was made to establish optimal conditions for a coupled phosphorylation.

The data presented are consistent with the view that phosphorylation is coupled to the transport of electrons which is mediated by hydroxylamine. It is conceivable, however, that the effects observed may be secondary to some interference of hydroxylamine with the endogenous metabolism of the extract used. The chemical might be thought to change the endogenous flow of electrons, thus canalizing them through a phosphorylating chain to cytochrome C. Several attempts have been made to demonstrate such an effect on the flow of electrons from DPNH, TPNH or succinate to cytochrome C, but without success. In this connection it is relevant to mention experiments which clearly demonstrate that reduction of cytochrome C by DPNH, TPNH and succinate under the conditions of the present experiments occurs without a concomitant phosphorylation (*Jysum & Jysum* unpubl. results). In the experiments reported in this paper the endogenous cytochrome phosphorylation was found to be unaffected by ageing of the extract (Table 3). It has been stated above that this is taken to indicate that the endogenous electrons, like those from the electron donors just mentioned, do not contribute to an electron transport phosphorylation.

The phosphorylation which is associated with the reduction of cytochrome C by hydroxylamine was uncoupled by ageing of the extract, by the addition of arsenate, and by the addition of fluoride ions, but not by DNP in the concentrations usually employed.

The effects of uncoupling agents on bacterial systems have recently been reviewed by *Ishikawa & Lehninger* (4). The lack of uncoupling by DNP has been reported from certain other, but not all, microbial systems. Fluoride ions do not uncouple oxidative phosphorylation in mitochondria. *Stekevitz & Potter* (10), however, found that if mitochondria are preincubated in the presence of  $F^-$ , they become inactive with respect to phosphorylation. These authors assume that  $F^-$  binds some compound or compounds necessary for mitochondrial phospho-

rylation. The lack of effect in short time experiments with mitochondria may indicate that the intact structures are to some extent protected against the fluoride effect. Findings by Pullman, Penefsky, Datta & Racker (9) that the intramitochondrial  $Mn^{++}$  activated ATP-ase to some extent was inhibited in the presence of  $0.02 M$  fluoride may also indicate an effect of this inhibitor on oxidative phosphorylation.

Whether the findings from the present experiments with meningococcal extracts indicate a true difference between the hydroxylamine-cytochrome C reductase system and other bacterial systems concerned with electron transport phosphorylation is not known.

The analysis of uncoupling agents has not been considered a main issue of the present investigation. The experiments reported were performed with the purpose of exploring the nature of the effects previously called "endogenous cytochrome phosphorylation" and "endogenous hydroxylamine phosphorylation" (Table 2). The results are taken to indicate that these effects are unrelated to an electron transport phosphorylation.

The P/O ratios calculated in the present paper are open to some discussion. A decisive point in this discussion is the endogenous reduction of cytochrome C. A calculation of hydroxylamine mediated reduction by subtracting the sum of endogenous and chemical reduction may result in too low values. It may be argued that the endogenous reduction is decreased in the presence of hydroxylamine, thus only unfavourably competing for the common pool of added cytochrome C. If so, the P/O ratios calculated may be too high.

The P/O ratios calculated in the present experiments are consistent with the conclusion that a maximum of two phosphorylations are coupled to the transport of each pair of electrons to cytochrome C from hydroxylamine.

#### SUMMARY

The reduction of mammalian ferricytochrome C which is mediated by hydroxylamine in the presence of extracts from *Neisseria meningitidis* is accompanied by a phosphorylation.

In these experiments the reoxidation of ferrocyclochrome C was prevented by an inhibition of the cytochrome oxidase with cyanide. The system was supplemented with labelled orthophosphate, ADP and  $Mg^{++}$ .

The phosphorylation connected with the reduction of cytochrome C from hydroxylamine has been analysed in relation to the endogenous uptake of inorganic phosphate.

P/O ratios have been calculated from measurements of the amounts of ferricytochrome C reduced and the orthophosphate taken up. The ratios calculated have been discussed in relation to the endogenous electron flow to cytochrome C.

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## INACTIVATION OF METHICILLIN, OXACILLIN, CLOXACILLIN, AND CEPHALOTHIN BY STAPHYLOCOCCAL PENICILLINASE

By

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The so called penicillinase-resistant penicillins are not quite unaffected by penicillinase. It was shown already in early reports on methicillin that this compound is inactivated by penicillinase produced by *B. cereus* (16) and several authors have found that methicillin, when present in high concentrations, is inactivated at an appreciable rate also by staphylococcal penicillinase (15, 18, 20). In concentrations which can be achieved in the human organism during its therapeutic use, methicillin is generally believed to be unaffected by staphylococcal penicillinase.

Consequently, the slightly lower susceptibility to methicillin of penicillinase producing as compared to penicillin sensitive staphylococci, observed by several authors, is not regarded as a manifestation of penicillinase activity (4).

The newer penicillins e.g. the isoxazolyl compounds, and some of the cephalosporins are less resistant than methicillin to the action of staphylococcal penicillinase and they are slowly inactivated also when present in 'therapeutic' concentrations. The fact that these compounds are much less active against a large than against a small inoculum of a penicillinase producing *Staphylococcus aureus*, and that non penicillinase producing staphylococci generally are more sensitive than penicillinase producing strains has been explained as a result of inactivation (4, 13).

Staphylococci resistant to the penicillinase resistant penicillins, whether naturally occurring or made resistant *in vitro*, have by most authors been found not to cause any inactivation of these penicillins. Stewart & Holt (21) however, isolated from clinical sources strains of *Staphylococcus aureus* resistant to the newer penicillins, which during growth inactivated isoxazolyl penicillins to a significant degree. Eriksen & Eriksen (7, 8) showed that also methicillin was inactivated in grow

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Semisynthetic penicillins were kindly supplied by H. Lundbeck & Co. A/S cephalothin by Eli Lilly and Co.

ing cultures of naturally occurring resistant strains. These findings were confirmed by *Ayliffe & Barber* (3) and *Knox & Smith* (14), who also demonstrated a significant inactivation of methicillin and cloxacillin in full grown cultures of highly penicillinase-producing strains of *Staphylococcus aureus* induced with methicillin or cloxacillin, irrespective of sensitivity to these penicillins.

The purpose of the present paper is to give a report of our continued studies on inactivation of methicillin caused by staphylococcal penicillinase under various experimental conditions, and of parallel studies with oxacillin, cloxacillin, and cephalothin.

## MATERIAL AND METHODS

The strains of *Staphylococcus aureus* used in these studies were as in previously reported experiments (8): 55974 a naturally occurring methicillin resistant strain 2719 a methicillin sensitive highly penicillinase producing strain and 326 a penicillin sensitive strain. The methicillin sensitive highly penicillinase producing strain 1634 which readily splits off penicillinase-negative mutants has been used in a few experiments.

Tests for inactivation of methicillin, oxacillin, cloxacillin and cephalothin were carried out in actively growing cultures as well as in full grown cultures. The medium used was nutrient broth pH 7.6. All experiments were carried out at 37° C.

Inactivation during active growth was tested in 5 ml cultures. In most experiments a number of separate cultures was used. In such cases all cultures were pipetted off from one bottle of medium to which drug and inoculum had just been added. The inoculum was in all experiments an overnight broth culture which was diluted with broth in the proportion 1:10 giving an initial number of staphylococci of 100-150  $\times 10^6$  per ml.

Full grown cultures were prepared in Erlenmeyer flasks with 50-100 ml of broth seeded with a middle sized inoculum. After incubation for 18 hours the antibiotic contained in a volume of 0.5-1 ml was added and aliquots of 5 ml were distributed in tubes to be used for testing inactivation.

Methicillin induced cultures were also large cultures grown in flasks. Induction was carried out in two different ways. In method 1 the culture was grown in broth containing 0.25  $\mu\text{g/ml}$  of methicillin. In method 2 methicillin was added to the 18 hour broth culture to give a concentration of 0.25  $\mu\text{g/ml}$ . The culture was then shaken for 6 hours at 37° C. In both cases the induced cultures were tested for inactivating properties exactly as described above, antibiotic being added and 5 ml aliquots being pipetted off.

At specified intervals a test tube was removed from the incubator and the culture fluid was immediately passed through a *Seitz* filter. The filtrate which was sterile and entirely free of penicillinase (9) could be kept for several hours at room temperature without any further loss of antibiotic activity. If stored for more than a few hours until testing for antibiotic activity could be carried out the filtrates were kept at 4° C.

Residual drug activity was estimated by the agar cup method described by *Yester dal* (22). The activity was calculated from dose response curves constructed from diameters of inhibition zones given by freshly prepared standard solutions of the particular penicillin.

The test organism was for the assay of oxacillin, cloxacillin and cephalothin *Staphylococcus aureus* 209 P. For the assay of methicillin a strain of *B. subtilis* was used.

In each experiment a tube with uninoculated broth containing the same concentration of drug as used in the cultures was incubated. At the end of the experiment also the control broth was *Seitz* filtered and assayed for drug activity. No loss of activity was observed in any experiment described in this paper.

## RESULTS

Inactivation of methicillin during growth of methicillin resistant strains of *Staphylococcus aureus* has been tested in several experiments and with a number of different strains. It was found that in broth with an initial concentration of 10  $\mu\text{g/ml}$  the activity after 24 hours incubation at 37° C was usually so small that no inhibition zone was seen when using staphylococcus 209 P as test organism. The results of an experiment with staphylococcus S 5974 in which the activity was tested every two hours are shown in Table 1. A gradual decline in activity was observed beginning after 6–8 hours incubation.

However, using cup plates seeded with staphylococcus 209 P concentrations of methicillin lower than 2.5  $\mu\text{g/ml}$  do not give any inhibition zones. Therefore, in later experiments a strain of *B. subtilis*, which is much more sensitive to the action of methicillin, has been used.

It was subsequently observed that inactivation in cultures of S 5974 sometimes proceeded at a significantly slower rate, possibly due to the fact that the culture was not very stable in regard to penicillinase-production: colonies of penicillinase-negative mutant strains often being found in significant numbers after spreading on agar plates (8). Therefore, a penicillinase producing strain was isolated from one single colony. In all later experiments this strain, which proved very stable, was used. It inactivated methicillin at a somewhat higher rate than did the strain used in the experiments shown in Table 1.

TABLE 1

*Inactivation of Methicillin during Growth of the Methicillin Resistant Staphylococcus aureus S 5974. Remaining Activity Tested after 6–22 Hours. Initial Concentration of Methicillin 10  $\mu\text{g/ml}$*

| Hours | Diameter of inhibition zone in mm |
|-------|-----------------------------------|
| 0     | 29                                |
| 6     | 29                                |
| 8     | 27                                |
| 10    | 25                                |
| 12    | 23                                |
| 14    | 21                                |
| 16    | 19                                |
| 18    | 17                                |
| 20    | 14                                |
| 22    | <10                               |

In Table 2 is shown the inactivation which took place during growth of *B. subtilis* with the methicillin sensitive

penicillinase producing staphylococcus 2719. For all compounds a significant inactivation was observed during growth of the resistant strain.

The sensitive strain caused inactivation of oxacillin and cloxacillin at a somewhat slower rate than did the resistant one. Also cephalothin was inactivated during growth of the sensitive strain. With the large inoculum used growth was obtained in all three compounds after 24–48 hours. In methicillin containing broth the sensitive strain did not grow and in this case inactivation proceeded at a very slow rate.

TABLE 2

*Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin during Growth of a Methicillin-Resistant (S 5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus*  
Initial Concentration of Each Antibiotic 10 µg/ml

| Remaining activity in µg/ml after incubation for 6–72 hours |             |      |           |      |             |      |             |      |
|---|-------------|------|-----------|------|-------------|------|-------------|------|
| Hours   | Methicillin |      | Oxacillin |      | Cloxacillin |      | Cephalothin |      |
|   | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 | S 5974      | 2719 |
| 6   | —           | —    | 3.4       | 5.0  | 2.8         | 4.4  | 10.0        | 10.0 |
| 8   | —           | —    | 0.6       | 3.6  | 1.7         | 3.6  | 6.4         | 8.0  |
| 10  | —           | —    | <0.3      | 1.9  | 0.4         | 2.1  | —           | —    |
| 12  | —           | —    | —         | 0.9  | <0.3        | 1.8  | 2.5         | 8.0  |
| 24  | 0.7         | 8.0  | —         | —    | —           | <0.3 | <0.3        | 2.0  |
| 48  | <0.3        | 8.0  | —         | —    | —           | —    | —           | <0.3 |
| 72  | —           | 5.0  | —         | —    | —           | —    | —           | —    |

TABLE 3

*Inactivation of Methicillin, Oxacillin and Cephalothin added to Non Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin-Sensitive (2719) Penicillinase Producing Staphylococcus aureus*  
Initial Concentration of Each Antibiotic 10 µg/ml

| Remaining activity in µg/ml after 12–120 hours |             |      |           |      |             |      |
|--|-------------|------|-----------|------|-------------|------|
| Hours  | Methicillin |      | Oxacillin |      | Cephalothin |      |
|  | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 |
| 12   | —           | —    | 10.0      | 10.0 | 10.0        | 10.0 |
| 24   | 10.0        | 9.0  | 6.5       | 6.5  | 7.5         | 7.5  |
| 48   | 5.0         | 3.8  | 0.5       | <0.3 | 1.3         | 0.9  |
| 72   | 3.2         | 2.5  | <0.3      | —    | <0.3        | <0.3 |
| 96   | 1.6         | 1.2  | —         | —    | —           | —    |
| 120  | 0.8         | 0.8  | —         | —    | —           | —    |

Table 3 shows the results of experiments in which the compounds were added to full grown cultures. For all three compounds tested inactivation took place at a very slow rate. The inactivating properties of the two different cultures were virtually identical.

The results of experiments with methicillin induced cultures are presented in Table 4–6. All four compounds were inactivated to a significant degree by induced cultures of both the methicillin-resistant and the methicillin sensitive staphylococcus, the cultures induced by method 2 being most active.

TABLE 4

*Inactivation of Methicillin Oxacillin Cloxacillin and Cephalothin Added to Methicillin Induced Broth Cultures of a Methicillin Resistant (S.5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus Induction by Growth in Methicillin Containing Broth (Method 1)*  
Initial Concentration of Each Antibiotic 10 µg/ml

Remaining activity in µg/ml after 1 & 2 hours

| Hours | Methicillin |      | Oxacillin |      | Cloxacillin |      | Cephalothin |      |
|-------|-------------|------|-----------|------|-------------|------|-------------|------|
|       | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 | S 5974      | 2719 |
| ½     | -           | -    | 2.5       | 4.4  | -           | -    | -           | -    |
| 1     | -           | -    | 1.2       | 2.8  | -           | -    | -           | -    |
| 2     | -           | -    | <0.3      | 0.9  | 2.1         | 4.0  | 10.0        | 10.0 |
| 3     | -           | -    | -         | 0.4  | 0.9         | 2.4  | 7.0         | 8.5  |
| 4     | -           | -    | -         | <0.3 | <0.3        | 1.3  | 4.6         | 5.0  |
| 6     | -           | -    | -         | -    | -           | <0.3 | <0.3        | 4.2  |
| 8     | -           | -    | -         | -    | -           | -    | -           | 1.0  |
| 12    | -           | -    | -         | -    | -           | -    | -           | <0.3 |
| 24    | 3.2         | 3.2  | -         | -    | -           | -    | -           | -    |
| 48    | 1.0         | 1.0  | -         | -    | -           | -    | -           | -    |
| 72    | <0.3        | <0.3 | -         | -    | -           | -    | -           | -    |

TABLE 5

(Method 4)  
Initial Concentration of Each Antibiotic 10 µg/ml

Remaining activity in µg/ml after 1 & 24 hours

| Hours | Methicillin |      | Oxacillin |      | Cloxacillin |      | Cephalothin |      |
|-------|-------------|------|-----------|------|-------------|------|-------------|------|
|       | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 | S 5974      | 2719 |
| ½     | -           | -    | 1.6       | 1.0  | 4.0         | 1.8  | -           | -    |
| 1     | -           | -    | 0.4       | <0.3 | 0.7         | <0.3 | 6.0         | 4.2  |
| 2     | -           | -    | <0.3      | -    | <0.3        | -    | 3.6         | 1.8  |
| 4     | -           | -    | -         | -    | -           | -    | <0.3        | <0.3 |
| 8     | 2.2         | 1.8  | -         | -    | -           | -    | -           | -    |
| 12    | 1.4         | 0.7  | -         | -    | -           | -    | -           | -    |
| 24    | <0.3        | <0.3 | -         | -    | -           | -    | -           | -    |

Oxacillin and cloxacillin were inactivated at a particularly high rate. With an initial concentration of 100 µg/ml inactivation was virtually complete after 1-2 hours with 100 µg/ml after 2-4 hours.

The slight differences observed between the inactivating properties of cultures of the methicillin-resistant and the methicillin-sensitive strain, respectively, were probably not significant.

In Table 7 are shown the results of an experiment in which the susceptibility to methicillin and cloxacillin of two penicillinase producing strains of *Staphylococcus aureus* has been compared.



The sensitive strain caused inactivation of oxacillin and cloxacillin at a somewhat slower rate than did the resistant one. Also cephalothin was inactivated during growth of the sensitive strain. With the large inoculum used growth was obtained in all three compounds after 24–48 hours. In methicillin-containing broth the sensitive strain did not grow and in this case inactivation proceeded at a very slow rate.

TABLE 2

*Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin during Growth of a Methicillin Resistant (S 5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus*  
Initial Concentration of Each Antibiotic 10 µg/ml

| Remaining activity in µg/ml after incubation for 6–72 hours |             |      |           |      |             |      |             |      |
|---|-------------|------|-----------|------|-------------|------|-------------|------|
| Hours   | Methicillin |      | Oxacillin |      | Cloxacillin |      | Cephalothin |      |
|   | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 | S 5974      | 2719 |
| 0   | —           | —    | 3.4       | 5.0  | 2.8         | 4.4  | 10.0        | 10.0 |
| 8   | —           | —    | 0.6       | 3.6  | 1.7         | 3.6  | 6.4         | 8.0  |
| 10  | —           | —    | <0.3      | 1.9  | 0.4         | 2.1  | —           | —    |
| 12  | —           | —    | —         | 0.9  | <0.1        | 1.8  | 2.5         | 8.0  |
| 24  | 0.7         | 8.0  | —         | —    | —           | <0.3 | <0.3        | 2.0  |
| 48  | <0.3        | 8.0  | —         | —    | —           | —    | —           | <0.3 |
| 72  | —           | 5.0  | —         | —    | —           | —    | —           | —    |

TABLE 3

*Inactivation of Methicillin, Oxacillin and Cephalothin added to Non Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin Sensitive (2719) Penicillinase Producing Staphylococcus aureus*  
Initial Concentration of Each Antibiotic 10 µg/ml

| Remaining activity in µg/ml after 12–120 hours |             |      |           |      |             |      |
|--|-------------|------|-----------|------|-------------|------|
| Hours  | Methicillin |      | Oxacillin |      | Cephalothin |      |
|  | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 |
| 12   | —           | —    | 10.0      | 10.0 | 10.0        | 10.0 |
| 24   | 10.0        | 8.0  | 6.5       | 6.5  | 7.5         | 7.5  |
| 48   | 5.0         | 3.8  | 0.5       | <0.3 | 1.3         | 0.9  |
| 72   | 3.2         | 2.5  | <0.3      | —    | <0.3        | <0.3 |
| 96   | 1.6         | 1.2  | —         | —    | —           | —    |
| 120  | 0.8         | 0.8  | —         | —    | —           | —    |

Table 1 shows the results of experiments in which the compounds were added to full grown cultures. For all three compounds tested inactivation took place at a very slow rate. The inactivating properties of the two different cultures were virtually identical.

The results of experiments with methicillin induced cultures are presented in Table 4. All four compounds were inactivated to a significant degree by induced cultures of both the methicillin resistant and the methicillin-sensitive staphylococcus, the cultures induced by method 2 being most active.

## DISCUSSION

The possible significance of inactivation by penicillinase for anti-staphylococcal activity and clinical effect of the so called penicillinase-resistant penicillins is a matter in dispute

Reports demonstrating that both methicillin and isoxazolyl penicillins are inactivated by penicillinase from *B. cereus* and when they are present in high concentrations also by staphylococcal penicillinase (15 16 18 20) did not cause any change in the commonly held view that these penicillins for all practical purposes are unaffected by penicillinase. Despite amply confirmed reports that isoxazolyl penicillins are less penicillinase-resistant than methicillin (15 18) some authors even regard all these penicillins as equally resistant to penicillinase (2)

The current view concerning penicillinase resistance of the new penicillins however must be fundamentally changed as a consequence of the recent findings that both methicillin and isoxazolyl penicillins are inactivated during growth of methicillin resistant staphylococci and that cultures of penicillinase-producing staphylococci whether methicillin resistant or not inactivate these penicillins when pre-induced with methicillin or cloxacillin (3 7 8 14 21)

The experiments reported in the present paper have fully confirmed that methicillin oxacillin and cloxacillin under certain experimental conditions are inactivated by staphylococcal penicillinase also when present only in therapeutic concentrations. Also the semi-synthetic cephalosporin cephalothin was inactivated at a significant rate.

The rate of inactivation particularly of the isoxazolyl penicillins was in some of our experiments so considerable that it appears highly probable that inactivation is a factor of importance for the action of these penicillins both *in vitro* and *in vivo*.

Experimental results published in a previous paper (8) indicated that penicillinase activity contributes significantly to the resistance of naturally occurring methicillin resistant strains of *Staphylococcus aureus*. However methicillin resistance is not only a result of penicillinase activity and at least some methicillin resistant strains of *Staphylococcus albus* isolated from clinical sources do not inactivate methicillin at all as shown by Kjellander *et al* (11 12). We have examined a few highly methicillin resistant strains and have found that even methicillin induced cultures of such strains do not inactivate methicillin.

Also for the *in vitro* activity against methicillin sensitive staphylococci however penicillinase-activity appears to be of some significance.

Thus the rather marked inoculum size effect observed with penicillinase producing staphylococci against both isoxazolyl penicillins and cephalosporins has been regarded as being due to inactivation by penicillinase (4 8).

Sabath & Finland (17) made a thorough study of the stability of methicillin and oxacillin in fluid medium inoculated with penicillinase

of the two penicillinase-producing strains was significantly lower than that of the non-penicillinase-producing strains. Also against methicillin the penicillinase-producing strains were somewhat less sensitive than the penicillinase-negative strains. The difference, however, was only evident when the figures relating to confluent growth were compared. In the case of oxacillin the difference in activity was approximately the same whether estimated from the observation of confluent growth or of complete growth inhibition

TABLE 6

*Inactivation of Methicillin, Oxacillin, Cloxacillin and Cephalothin Added to Methicillin-Induced Broth Cultures of a Methicillin Resistant (S 5974) and a Methicillin-Sensitive (2719) Penicillinase-Producing Staphylococcus aureus Induction by Shaking Broth Cultures for 6 Hours after Addition of Methicillin (Method 2).*

*Initial Concentration of Each Antibiotic 100 µg/ml*

| Remaining activity in µg/ml after 1/2, 48 hours |             |      |           |      |             |      |             |      |
|---|-------------|------|-----------|------|-------------|------|-------------|------|
| Hours   | Methicillin |      | Oxacillin |      | Cloxacillin |      | Cephalothin |      |
|   | S 5974      | 2719 | S 5974    | 2719 | S 5974      | 2719 | S 5974      | 2719 |
| 1/2   | -           | -    | 32        | 22   | -           | -    | -           | -    |
| 1   | -           | -    | 5.0       | 0.4  | 25          | 8.0  | -           | -    |
| 2   | -           | -    | 0.6       | <0.3 | 9.0         | 0.9  | -           | -    |
| 3   | -           | -    | <0.3      | -    | 2.3         | <0.3 | -           | -    |
| 4   | -           | -    | -         | -    | <0.3        | -    | -           | -    |
| 8   | 25          | 25   | -         | -    | -           | -    | 70          | 70   |
| 12  | 18          | 16   | -         | -    | -           | -    | 60          | 50   |
| 24  | 1.6         | 1.6  | -         | -    | -           | -    | <0.3        | <0.3 |
| 48  | <0.3        | <0.3 | -         | -    | -           | -    | -           | -    |

TABLE 7

*Sensitivity to Methicillin and Oxacillin of Penicillinase-Producing and Non-Penicillinase-Producing Strains of Staphylococcus aureus, Estimated on Solid Medium Inoculated with 0.1 ml Undiluted Broth Culture (100-150 × 10<sup>6</sup> Bacteria)*

|  | Methicillin  | Oxacillin     |
|--|--------------|---------------|
| Penicillinase-producing<br><i>Staphylococcus aureus</i> 2719                       | 1.25 (1.25)  | 0.312 (1.25)  |
| Penicillinase producing<br><i>Staphylococcus aureus</i> 1634                       | 1.25 (1.25)  | 0.312 (0.625) |
| Penicillinase-negative strain<br>derived from<br><i>Staphylococcus aureus</i> 1634 | 0.625 (1.25) | 0.039 (0.156) |
| Penicillinase-sensitive<br><i>Staphylococcus aureus</i> 326                        | 0.625 (1.25) | 0.039 (0.078) |

For each strain the first figure gives the highest concentration showing confluent growth. The figure in brackets shows the highest concentration in which growth occurred at all.

ly inactivated by staphylococcal penicillinase which is produced in large amounts also in non induced cultures

The penicillinase-resistant penicillins are much more active inducers of penicillinase than is penicillin G a property presumably related to their penicillinase resistance (15 16 18 20) It appears probable that these penicillins under certain conditions will be active inducers also *in vivo*

As optimal induction of staphylococcal penicillinase occurs only when the bacteria are actively multiplying (19) it is less likely that induction will take place if the staphylococcus is methicillin sensitive because growth of the bacterial cells will be inhibited by the antibiotic If tissue concentration of antibiotic is very low which may happen for various reasons of course induction may be supposed to take place also when the staphylococci are methicillin sensitive

Apliffe & Barber (3) and Knox & Smith (14) have emphasized that inactivation of methicillin and cloxacillin even in induced cultures is infinitesimal as compared with the inactivation of penicillin II But this fact does not necessarily mean that inactivation has no practical implications Inactivation may very well be a significant factor interfering with the therapeutic effect of penicillinase resistant penicillins and cephalothin in infections with methicillin resistant staphylococci and perhaps if induction is allowed to take place during an initial phase with very low tissue concentrations also in infections caused by methicillin sensitive staphylococci

#### SUMMARY

Inactivation of some penicillinase resistant penicillins and cephalothin by staphylococcal penicillinase has been tested under various experimental conditions During growth of a methicillin resistant strain of *Staphylococcus aureus* a significant inactivation was observed The isoxazolyl penicillins were inactivated also during growth of a meth

Results of some comparative experiments with penicillinase producing and non penicillinase producing strains of *Staphylococcus aureus* indicate that penicillinase activity contributes to the *in vitro* resistance of penicillinase producing staphylococci against both oxacillin and methicillin

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producing staphylococci. They observed a secondary outgrowth of staphylococci with unchanged sensitivity to methicillin and oxacillin coincident with a reduction of antibiotic concentration to subinhibitory values.

Klein *et al* (13) suggested that the destruction of oxacillin accounts for differences in sensitivity observed between penicillinase-producing and penicillin-sensitive strains of *Staphylococcus aureus*.

Abu-Nassar *et al* (1), however, found no relationship between penicillinase production and the resistance of staphylococci to methicillin and oxacillin.

Experiments presented in this paper (Table 7) have demonstrated a considerable difference in sensitivity to oxacillin between penicillinase-producing and non-penicillinase-producing staphylococci. Also against methicillin are the penicillinase-producers more resistant than the penicillinase-negative strains.

Particularly noteworthy is the difference in sensitivity between the penicillinase-producing staphylococcus 1634 and the penicillinase negative strain derived from it. This mutant strain was used also in previously published experiments and was shown to be fully sensitive to penicillin G (8). It appears from Table 7 that its susceptibility also against oxacillin and methicillin is the same as that of an ordinary penicillin-sensitive staphylococcus. The experimental results obtained with this strain appears to carry a strong evidence in favour of the hypothesis that penicillinase activity contributes to the *in vitro* resistance of penicillinase-producing staphylococci against both oxacillin and methicillin.

Sabath & Finland (17) have suggested that inactivation may account for difficulties observed in some cases in eradicating staphylococci during methicillin therapy. Persistence of staphylococci during treatment with the new penicillins has been observed also by other authors (5).

Inactivation of methicillin in uninduced cultures of a methicillin-sensitive staphylococcus as observed by Sabath & Finland and as demonstrated also in the present paper (Table 2) proceeds at a very slow rate. Thus, judging from experiments of this kind, it does not seem probable that inactivation of methicillin in the presence of methicillin-sensitive staphylococci can be a factor interfering with successful therapy.

If, however, induction of penicillinase formation takes place *in vivo* inactivation might be supposed to be a significant factor, in view of the high rate at which methicillin-induced cultures inactivated all compounds tested in the present investigation.

Several authors have suggested that induced formation of staphylococcal penicillinase may take place *in vivo* during treatment with penicillin G, but its occurrence has never been proved (6, 10). Possibly, induction is not very important because penicillin G is extremely rapid-

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## THE ISOLATION OF SALMONELLA IN A SWEDISH WATER COURSE (THE RIVER FYRIS)

*1 Isolation by Various Filter Methods and the Swab Technique  
According to Moore<sup>1</sup>*

By

ABERRA DEMISSIE

Received 20 ix 64

In isolating enteropathogenic salmonella from different kinds of water, such as tap water, well water and river water, different methods have come into use

The committee on Bathing Beach Contamination (1959) obtained positive results by filtering large volumes of sea water through Sterimat filter of porosity FCB and by culturing the pads

Collet and her collaborators (1953) have successfully isolated *S. typhi* from a well located near the home of a typhoid carrier by filtering varying quantities of water through membrane filter

Ljutov (1954) employed Hyphlo supercel (the fossil remains of diatoms) and a textile fabric in demonstrating salmonella bacteria in water by filtering large quantities

A simple technique for the location of enteropathogenic bacteria in towns by means of serial sewage examinations was described by Moore (1948, 1950)

In the comparative studies presented here, the above filters and a modified Moore's swab were used to isolate enteropathogenic salmonella from a Swedish watercourse (the river Fyris) during a four months period (May-August 1963)

### MATERIAL AND METHODS

#### *1 The Water Course*

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<sup>1</sup> This investigation was made possible through a scholarship grant from NIB (Nämnden för internationellt bistånd) and financial assistance from the Department of Bacteriology

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A simple technique for the location of enteropathogenic bacteria in towns by means of serial sewage examinations was described by Moore (1948 1950)

In the comparative studies presented here the above filters and a modified Moore's swab were used to isolate enteropathogenic salmonella from a Swedish watercourse (the river Fyris) during a four months period (May-August 1963)

### MATERIAL AND METHODS

#### *A The Water Course in Question and Plan of Investigation*

The River Fyris originated from a small lake in the North of Uppland collected water from smaller streams passed through Uppsala and fell into lake Mälaren

Three sites Jarnbron Harbour and Flottsund referred to as I II and III respectively hereafter were chosen for the present work Site I was located in the upper

<sup>1</sup> This investigation was made possible through a scholarship grant from NIB (Nämnden för internationellt bistånd) and financial assistance from the Department of Bacteriology



## II Experimental

Preliminary tests with water artificially infected with *S typhi* murium were designed to evaluate the sensitivity of the filtration methods. The test organism *S typhi* murium had been isolated from the river. The bacteria were grown over night at 37° C and diluted to  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  in saline. The viable count was determined by plating 0.3 ml of each dilution on a large Endo plate. One ml of each dilution was added to three litres of river water. The water used for the  $10^{-9}$  dilution had been sterilized but non-sterilized water was used for the two other dilutions. 10 experiments were undertaken for each dilution.

## RESULTS

### The General Character of the Various Filter Methods

In using the membrane filter in filtering polluted river water difficulties were encountered. It became evident that the membrane filter was too slow, requiring 4-5 hours to filter a three-litre-sample of specimen due to the blocking effect of particles in the impure river water.

The use of this filter, was therefore abandoned at an early stage of the investigation.

It is seen from Table 1 that about 13 per cent of the organisms present in the original sample passed through when 0.5 gr of supercel was used, compared to about 6 per cent when the supercel material was raised to 1.0 gr. The larger amount of filter material made the filtration more difficult as in the case of the membrane filter. Three litres of river water was filtered in about ½ hour and 10 litres in about three hours.

TABLE 1  
MPN Test on the Number of Coliform Bacteria Passing through  
Supercel- and Seitz Filters

| No of experiments | Filter method   | Coliform 100 ml |              | Plate count<br>37° C |
|-------------------|-----------------|-----------------|--------------|----------------------|
|                   |                 | 37° C           | 41° C        |                      |
| 9                 | Untreated       | 8444 ± 950.8    | 1789 ± 847.6 | 1528 ± 896.1         |
| 9                 | 0.5 gr supercel | 1078 ± 777.8    | 478 ± 705.7  | 178 ± 87.6           |
| 9                 | 1.0 gr supercel | 522 ± 440.5     | 144 ± 389.9  | 122 ± 93.2           |
| 9                 | Seitz           | —               | —            | —                    |

The results obtained by the Seitz filter were found to be similar to those obtained by the supercel method. Filtration of large quantities (9) took close to two hours. While some bacteria escaped through the Seitz filter (Table 1).

Preliminary tests in which the supercel and the Seitz filter were used in combination, first filtering through supercel (0.5 gr) and then through Seitz filter, showed no passing through of the bacteria. The time required to filter 9 litres of raw river water was 2 hours.

### The Isolation of Salmonella Bacteria

The isolation of Salmonella is demonstrated in Table 2. 20 specimens of water were filtered through supercel filter and *S typhimurium* was

found in one specimen 5 filtrations through Seitz filter were negative 61 swabs were examined and three salmonella strains were isolated from the river and five from the manholes

TABLE 2

*The Isolation of Salmonella Bacteria in Water From the River Fyris and in Sewagewater Manholes of the Sewage System of Uppsala*

| Technique for isolation | Source  | No of specimens | Pos no of specimens | Date of isolation | Types of strains isolated |
|-------------------------|---------|-----------------|---------------------|-------------------|---------------------------|
| Swab                    | Harbour | 40              | 1                   | 10 6              | <i>S newport</i>          |
|                         |         |                 | 1                   | 23 6              | <i>S paratyphi B</i>      |
|                         |         |                 | 1                   | 29 7              | <i>S blockley</i>         |
| Swab                    | Manhole | 21              | 3                   | 27 8              | <i>S blockley</i>         |
|                         |         |                 |                     | 29                | <i>S blockley</i>         |
|                         |         |                 |                     | 10 9              | <i>S blockley</i>         |
|                         |         |                 | 1                   | 18 9              | <i>S typhimurium</i>      |
|                         |         |                 | 1                   | 10 9              | <i>S enteritidis</i>      |
| Supræpel Seitz          | Harbour | 20              | 1                   | 23 6              | <i>S typhimurium</i>      |
|                         | Harbour | 5               | 0                   |                   | —                         |

TABLE 3

*An Epidemiological Study of a Case of S blockley by Isolation of Bacteria from Stool in Manhole of the Sewage System and Water of the River Fyris at the Outlet from the Sewage Treatment Plant*

| Date | Isolation of <i>S Blockley</i> |         |             |
|------|--------------------------------|---------|-------------|
|      | Faeces                         | Manhole | River water |
| 13 5 | pos +                          |         |             |
| 5 6  | neg —                          |         | neg —       |
| 17 6 | pos +                          |         | neg —       |
| 29 6 | pos +                          |         | neg —       |
| 29 7 | pos +                          |         | neg —       |
| 23 8 | pos +                          |         | pos +       |
| 3 9  | neg —                          | pos +   |             |
| 12 9 | neg —                          | pos +   |             |
| 16 9 | neg —                          | pos +   |             |
| 20 9 | neg —                          | neg —   |             |

### Epidemiological Survey

One of the salmonella strains that was isolated from the river, at site II was *S blockley* as shown in the Table 2. Only one case caused by this type of salmonella was known in the town.

The data are given in Table 3. The strain was isolated from faeces on May 13 1963 and from a hospital on May 12 1963. Isolations of

positive at several occasions be

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| Technique for isolation | Source  | No of specimens | Pos no of spec in env | Date of isolation | Types of strains isolated |
|-------------------------|---------|-----------------|-----------------------|-------------------|---------------------------|
| Swab                    | Harbour | 40              | 1                     | 10.6              | <i>S. newport</i>         |
|                         |         |                 | 1                     | 25.6              | <i>S. paratyphi B</i>     |
|                         |         |                 | 1                     | 29.7              | <i>S. blockley</i>        |
| Swab                    | Manhole | 21              | 3                     | 27.8              | <i>S. blockley</i>        |
|                         |         |                 |                       | 29                | <i>S. blockley</i>        |
|                         |         |                 |                       | 10.9              | <i>S. blockley</i>        |
|                         |         |                 | 1                     | 18.9              | <i>S. typhimurium</i>     |
|                         |         |                 | 1                     | 10.9              | <i>S. enteritidis</i>     |
| Supercoiled Seitz       | Harbour | 20              | 1                     | 25.6              | <i>S. typhimurium</i>     |
|                         | Harbour | 5               | 0                     |                   | —                         |

TABLE 3

*An Epidemiological Study of a Case of S. blockley by Isolation of Bacteria from Stool, a Manhole of the Sewage System and Water of the River Fyris at the Outlet from the Sewage Treatment Plant*

| Date | Isolation of <i>S. Blockley</i> |         |             |
|------|---------------------------------|---------|-------------|
|      | Faeces                          | Manhole | River water |
| 13.5 | pos +                           |         | neg —       |
| 5.6  | neg —                           |         | neg —       |
| 17.6 | pos +                           |         | neg —       |
| 29.6 | pos +                           |         | neg —       |
| 29.7 | pos +                           |         | pos +       |
| 25.8 | pos +                           | pos +   |             |
| 3.9  | neg —                           | pos +   |             |
| 12.9 | neg —                           |         |             |
| 16.9 | neg —                           | pos +   |             |
| 20.9 | neg —                           | neg —   |             |

### Epidemiological Survey

One of the salmonella strains that was isolated from the river at site II was *S. blockley* as shown in the Table 2. Only one case caused by this type of salmonella was known in the town.

The data are given in Table 3. The strain was isolated from faeces on May 13 1964 and the patient was admitted to the infectious disease hospital on May 17. The patient returned home on June 5. Control examinations of faeces were, however, positive at several occasions be

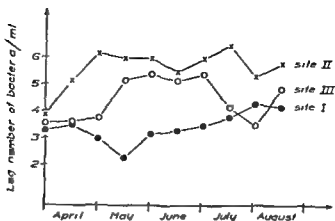


Fig 2

Number of coliform in the river Ivaris during the spring and summer 1963

tween June 17 and August 25. She was thus a healthy carrier and was readmitted for treatment to the hospital on August 28. Faeces became negative on September 3, and remained negative in four specimens, the last one examined on September 20.

*Salmonella Blockley* was found in water from site II (see Fig 1) on July 29, which finding started the examinations of the manholes. Thus *S. blockley* was isolated from a manhole adjacent to the house in which this patient lived on August 25, September 3, and 16. An examination on September 20, was negative.

The same manhole was positive for *S. enteritidis* as well as *S. typhimurium* of the same phage type as the *S. typhimurium*, which had been isolated from the river almost three months earlier. The strains could not be related to any known carrier.

The additional strains which had been isolated from the river, were *S. newport* and *S. paratyphi B*. They were not found in the sewage system. No carrier of *S. newport* was known in the town. An extensive epidemic caused by this strain, however, had occurred one year earlier. A patient with *S. paratyphi B* infection had been living in the town shortly before the time at which the strain was isolated from the river.

**The coliform test.** The results of the MPN estimation of the coliform density are presented in Fig 2. As may be seen from the figure, there was variation in the number of bacteria from the time the test began on April 17 to the time the work was concluded on August 27, 1963. The number of coliform bacteria increased slightly the first fortnight in specimens from I and III but II showed a 20 fold increase. The increase was from 2000 coliform/100 ml, 8000 coliform/100 ml and 3000 coliform/100 ml to 3000 coliform/100 ml, 150 000 coliform/100 ml and 4000 coliform/100 ml for I, II and III respectively. The peak of the coliform density for II and III was reached during the summer with 1 500 000 coliform/100 ml and 50 000 coliform/100 ml respectively. The coliform content of site I showed less variations.

TABLE 4

*Dilution Experiment with S typhimurium in Water from the River Ffris Using the Supercel Technique and Seitz Filter*

| No of experiments | Dilution         | No of pathogenic bacteria per ml | Volume of water ml | No of positive experiments |       |              |
|-------------------|------------------|----------------------------------|--------------------|----------------------------|-------|--------------|
|                   |                  |                                  |                    | Supercel filters           |       | Seitz filter |
|                   |                  |                                  |                    | 0.5 gr                     | 10 gr |              |
| 10                | 10 <sup>-1</sup> | 159                              | 3000               | 10                         | 10    | 10           |
| 10                | 10 <sup>-2</sup> | 26                               | 3000               | 8                          | 10    | 10           |
| 10                | 10 <sup>-3</sup> | <1                               | 3000               | 0                          | 3     | 5            |

### *Dilutions Experiments*

The results of the dilution experiments are presented in Table 4. In dilution 10<sup>-1</sup> containing about 159 *S typhimurium* per ml it was possible to recover the test organism irrespective of the amount of supercel whether 0.5 or 10 gr was used. The test organism was also recovered by the Seitz filter. The result with the 10<sup>-2</sup> dilution however, was somewhat different. The test organism was recovered in all 10 trials when 10 gr of supercel was used but only in 8 trials with 0.5 gr of supercel. The Seitz filter gave positive results in all 10 tests.

The water sample was sterilized before adding the test bacteria in a dilution of 10<sup>-3</sup>. This dilution gave no growth of bacteria on plates. Calculated from the lower dilutions the number of bacteria should have been 1.3 bacteria per ml. *S typhimurium* was recovered 3 times when 10 gr of supercel was used against none when the weight of this material was lowered to 0.5 gr. The Seitz filter recovered the bacteria in one half of the experiments.

### DISCUSSION

The use of the swab according to Moore had proven itself as an effective tool in epidemiological surveys on river and sewage water. *Salmonella* bacteria could be isolated in the river Ffris and in the related sewage system. On one occasion an infection of *Salmonella Blockley* was the only case known in the town. Bacteria were isolated simultaneously from faeces of the case, the sewage system and the river. The strain remained in the sewage system for about a fortnight longer than it could be isolated from the patient. The ordinary swab technique did not give quantitative results which might be a weakness in some instances. There is a possibility however utilized by Moore to get such results by passing the water through a device measuring the volume of passing water.

In filtration tests on larger volumes of polluted water the result was influenced by the size of the pores. There was naturally enough an inverse relationship between the filtration time and the capacity of the filter to retain pathogenic bacteria. Due to these circumstances the

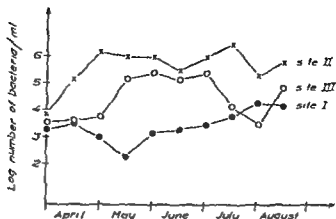


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The same manhole was positive for *S. enteritidis* as well as *S. typhimurium* of the same phage type as the *S. typhimurium*, which had been isolated from the river almost three months earlier. The strains could not be related to any known carrier.

The additional strains which had been isolated from the river, were *S. newport* and *S. paratyphi B*. They were not found in the sewage system. No carrier of *S. newport* was known in the town. An extensive epidemic caused by this strain, however, had occurred one year earlier. A patient with *S. paratyphi B* infection had been living in the town shortly before the time at which the strain was isolated from the river.

**The coliform test.** The results of the MPN estimation of the coliform density are presented in Fig 2. As may be seen from the figure, there was variation in the number of bacteria from the time the test began on April 17 to the time the work was concluded on August 27, 1963. The number of coliform bacteria increased slightly the first fortnight in specimens from I and III but II showed a 20-fold increase. The increase was from 2000 coliform/100 ml, 8000 coliform/100 ml and 3000 coliform/100 ml to 3000 coliform/100 ml, 150 000 coliform/100 ml and 4000 coliform/100 ml for I, II and III respectively. The peak of the coliform density for II and III was reached during the summer with 1 500 000 coliform/100 ml and 50 000 coliform/100 ml respectively. The coliform content of site I showed less variations.

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## LACK OF CHICK ALLANTOIC ANTIGEN IN INFLUENZA VIRUS PASSED IN CHICK KIDNEY MONOLAYERS

By

G ROLLA

Received 23 iv 64

The influenza virus haemagglutination inhibiting antibody produced after injection of normal host material, was first reported by *Knight*, and further investigated by *Harboe* (6, 1, 2, 3, 4) *Strandli*, *Mortenson-Egnund* & *Harboe* have recently published a method for the further purification of this antigen from normal chick allantoic fluid (8) A chemical analysis of the antigen showed it to be an acid mucopolysaccharide

The experiments described in the present report intend to find out whether influenza virus propagated in chick cell monolayers is inhibited by antiserum against chick allantoic material This antiserum inhibits viruses grown in the allantoic sac of chick embryos

In an additional experiment influenza virus grown in duck embryos was treated with normal chick allantoic fluid and subsequently tested for the presence of normal chick allantoic antigen

### MATERIALS AND METHODS

**Virus** The influenza strains A/PR8 and B/Lee were used The strains were grown in the chick embryo duck embryo or chick kidney cell monolayers They were purified by adsorption to and subsequent elution from

After inoculation of virus medium was changed every with virus the monolayers virus suspension was then for 90 minutes at 37° C The After four to five days the tubes were tested for



supercel filter using 0.5-1 gr of the material was the most suitable of the filters tested in epidemiological work on *Salmonella*. The supercel filter also showed its good quality in the dilution experiments with *S. typhimurium*. The pore-size of the steremat- and Seitz filters was too small. The bacteria were retained, but the filters were blocked by the impurities of the water, and the filtration took considerable time. There was a feasible prospect of using the supercel- and Seitz filters in combination.

The sewage system of Uppsala included a sewage treatment plant, which could apparently be passed by the *Salmonella* bacteria. The reason was not known. It could be seen that the number of coliform bacteria in the river varied considerably from time to time. The peak occurred during the summer months when the flow of water of the river had slowed down. *Salmonella* bacteria were found, in the river water during the whole summer.

#### SUMMARY

In a four months period, May-August 1963, a survey was conducted as to the occurrence of *salmonella* in the water of Fyrisån. Different methods were tested. The results of a modified swab technique according to Moore and of filtration through Membrane (MF), supercel and Seitz-filter was compared. The following strains of *salmonella* were isolated: *S. newport*, *S. typhimurium*, phage type NS, *S. paratyphi B*, phage type 3A1 var 2, *S. blockley* and *S. enteritidis*. The modified swab technique gave the highest number of positive results. One of the strains, *S. blockley*, was traced back to a manhole located in the vicinity of the only patient, who was known to be carrier of this strain in Uppsala at that time.

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The influenza virus haemagglutination-inhibiting antibody produced after injection of normal host material, was first reported by *Knight*, and further investigated by *Harboe* (6, 1, 2, 3, 4) *Strandli, Mortensson-Egnund & Harboe* have recently published a method for the further purification of this antigen from normal chick allantoic fluid (8) A chemical analysis of the antigen showed it to be an acid mucopolysaccharide

The experiments described in the present report intend to find out whether influenza virus propagated in chick cell monolayers is inhibited by antiserum against chick allantoic material This antiserum inhibits viruses grown in the allantoic sac of chick embryos

In an additional experiment influenza virus grown in duck embryos was treated with normal chick allantoic fluid and subsequently tested for the presence of normal chick allantoic antigen

### MATERIALS AND METHODS

*Virus* The influenza strains A/PR8 and B/Lee were used The strains were grown in  
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except it contained only 2 per cent calf serum The medium was changed every 24 hours during the growth period Before inoculation with virus the monolayers were washed three times with Hanks solution 0.1 ml of virus suspension was then added to each tube which was left for adsorption for 90 minutes at 37° C The culture was washed three times in Hanks solution and  
After four  
Haemaggl  
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was treated with five volumes of cholera filtrate (Philips Duphar) to destroy normal inhibitors (9) Serum haemagglutinin was adsorbed with 10 per cent fowl red cells

## RESULTS

### 1 Influenza Virus Passed in Chick Kidney Tissue Culture

A/PR8 was passed 4 times and B/Lee 5 times in chick kidney cells. The red cell eluates of the viruses were tested with rabbit antiserum against host material. No inhibition could be demonstrated. The results are given in Table 1.

TABLE 1  
*Haemagglutination Inhibition Test Showing The Absence of Normal Chick Allantoic Antigen in Tissue Culture Grown Viruses*

| Virus antigen                                    | Tested with antiserum to B/Lee | Tested with antiserum to A/PR8 |
|--|--------------------------------|--------------------------------|
| A/PR8 from the chick allantoic sac               | 48                             | 2000                           |
| B/Lee from the chick allantoic sac               | 1000                           | 96                             |
| A/PR8 propagated in chick kidney cell monolayers | <6                             | 1500                           |
| B/Lee propagated in chick kidney cell monolayers | 2000                           | <6                             |

TABLE 2  
*Haemagglutination Inhibition Tests for Normal Chick Allantoic Antigen in Purified Duck Embryo Grown Viruses Treated with Normal Chick Allantoic Fluid*

| Virus antigen  | Tested with antiserum to B/Lee | Tested with antiserum to A/PR8 |
|--|--------------------------------|--------------------------------|
| A/PR8 from the duck allantoic sac (9 passage)  | <6                             | 1500                           |
| B/Lee from the duck allantoic sac (8 passage)  | 2000                           | <6                             |
| Purified A/PR8 from the duck allantoic sac treated with normal chick allantoic fluid for |                                |                                |
| 2 hours at room temp   | <6                             | 1500                           |
| 24 hours at 5° C   | <6                             | 1500                           |
| Purified B/Lee from the duck allantoic sac treated with normal chick allantoic fluid for |                                |                                |
| 2 hours at room temp   | 2000                           | <6                             |
| 24 hours at 5° C   | 2000                           | <6                             |

The table shows that antiserum to B/Lee grown in the chick allantoic sac moderately inhibits allantoic A/PR8 and that antiserum to allantoic A/PR8 inhibits allantoic B/Lee. This inhibition of unrelated virus is caused by the antibody to host material (6, 2, 3, 4, 5). It is further seen from the table that virus propagated in chick tissue cultures was not inhibited by antibody to chick allantoic material.

### 2 Influenza Virus Passed in Duck Embryos and Treated with Normal Chick Allantoic Fluid

After several passages, red cell eluates were prepared and tested for presence of normal chick allantoic antigen. As this test thus gave no

evidence for the presence of this antigen the purified duck embryo viruses were transferred to normal chick allantoic fluid and left for two hours at room temperature or 24 hours at 5° C. The purification procedures were then performed once more and the viruses tested for the presence of normal chick allantoic antigen. The results are given in Table 2.

The table shows that viruses cultured in the duck allantoic sac were not inhibited by antibody against chick allantoic fluid even when the viruses had been pretreated with chick allantoic fluid.

### DISCUSSION

It has been shown that the normal chick allantoic antigen is present in preparations of influenza virus from the chick allantoic sac but not in duck allantoic sac or mouse lung cultured virus (3). Cross reacting antigens have been demonstrated in duck and turkey normal allantoic fluids but not in chick embryo viscera or guinea pig kidney (4).

The first experiments described in this report show that no chick allantoic antigen demonstrable in the HI test, is present in the viruses propagated in chick kidney cells.

In order to see if allantoic antigen material was attached to mature virus particles and thus might give an explanation to the phenomenon of the haemagglutination inhibition of antiserum to host material the duck allantoic viruses were treated with chick allantoic fluid. The subsequent HI test showed however that no such attachment took place.

### SUMMARY

- 1 The haemagglutinin of influenza virus grown in chick embryo kidney tissue culture was not inhibited by antiserum against chick allantoic material.
- 2 Antiserum to chick allantoic material did not inhibit the haemagglutinin of influenza virus grown in the duck allantoic sac and treated with normal chick allantoic fluid.

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# THE INCIDENCE OF COMPLEMENT-FIXING ANTIBODIES TO THE RESPIRATORY SYNCYTIAL VIRUS IN SERA FROM DANISH POPULATION GROUPS AGED 0-19 YEARS

By

ALLAN HØRSLETH and MOGENS VØLKERT

Received 11 iv 64

Since its detection in 1956 (3, 4) as a cause of human respiratory disease the Respiratory Syncytial (RS) virus has been repeatedly and firmly established as an important aetiological factor in severe respiratory infections in infants and children in the United States (1, 2, 5, 11, 14) in Great Britain (9, 17, 18), and in Australia (13). In adults it only seems to give infections resembling the common cold (7, 10, 12).

Since in one major study (8) it was found to be the most important single aetiological factor in acute respiratory illness in children less than eight years of age, and in view of the high percentage of sera from different age groups in England found to contain antibodies to this virus (15), it seemed to be of value to obtain an estimation of the antibodies present in Danish population groups.

## MATERIAL AND METHODS

Blood samples collected from all over Denmark during a one year period, blood samples from children with acute respiratory diseases admitted to hospitals in Copenhagen during the same period, and a number of sera stored in this department during six consecutive years, constitute the material.

Sera examined: a) 1324 single sera taken during the year August 1962 to July 1963. The number corresponds to an annual 57-68 patients at ages from 1 to 19 years plus 58 from the 0-5 months age group and 57 from the 6-11 months age group. 661 of the sera were collected from August to December 1962 and 663 from February to July 1963.

All the blood specimens were sent to the Treponematoses Department at the Statens Seruminstitut (for Wasserman testing). 79 were collected in general practice and the rest came from patients admitted to hospitals (both medical and surgical departments).

b) 151 paired sera which had been tested in the Influenza Department at this

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b) 151 paired sera which had been tested in the Influenza Department at this

The authors are indebted to Dr R M Chanock (Bethesda, U.S.A.)



Institute for complement fixing antibodies against influenza and adenovirus during the period September 1962 to June 1963

c) 268 single sera examined in this department by means of the psittacosis complement fixation test during the years 1957 to 1962. The majority of these sera were from the Copenhagen area.

All the specimens were heated for 30 minutes at 56° C and stored at -20° C until examined.

**Virus strain.** The Long strain of the RS virus, kindly provided by Dr R. M. Chanock, National Institutes of Health, Bethesda, U.S.A., was used throughout.

**Tissue culture.** HeLa cells grown in Roux bottles in Hanks medium containing 10 per cent horse serum and 0.5 per cent lactalbumin. One hundred units of penicillin and 100 micrograms of streptomycin per ml were added to all the media. The maintenance medium used in the preparation of complement fixing antigen was the same as in the assay of virus titres, except that it contained only 5 per cent horse serum.

**Preparation of antigen.** Roux bottles seeded three days previously with seven million cells per bottle were inoculated with 8-10 ml of maintenance medium containing approximately  $10^{7.0}$  TCD<sub>50</sub> RS virus per ml. When more than 90 per cent of the cells in the monolayer showed cytopathology (40-50 hours after inoculation), the bottles were frozen and thawed three times. The harvested cells plus medium were centrifuged at 2000 r.p.m. for 30 minutes and the supernatant fluid used as the antigen. The antigen was measured by the usual box titration and comparison of different lots of antigen was always made on the same day against the same serum.

Chanock *et al.* (3) showed that ultracentrifugation of infectious fluid for several hours failed to diminish the potency of the soluble Cf antigen in the supernatant fluid. In our experiments it was found that ultracentrifugation for one hour (Spinco swinging bucket 391) at 30 000 r.p.m. usually doubled the titre of the antigen present in the supernatant fluid after the preliminary low speed centrifugation. This was always accompanied by a decrease in the anticomplementary activity of the antigen thus indicating that ultracentrifugation probably caused removal of anticomplementary factors. By means of ultrafiltration (with a 1 kB filter type 6300 A) the titre of the antigen present in the ultraconcentrate was doubled when this fraction consisted of 15-20 per cent of the original volume, but no increase in titre was seen when the ultraconcentrate comprised more than 30 per cent of the original volume.

**The complement fixation test.** Serum dilutions two units of antigen and two units of complement each in amounts of 0.1 ml were mixed and kept overnight at 4° C for fixation. After addition of the haemolytic system (two units of haemolysin 0.1 ml and a two per cent sheep erythrocyte suspension 0.1 ml) the tubes were placed in a water bath at 37° C for one hour. Reading was made after further 30 minutes at room temperature. The end point of the titration was read as the last tube with at least 50 per cent complement fixation. Normal cell antigens and positive as well as negative sera were included as controls in all test series.

## EXPERIMENTAL

### *Incidence of Complement-Fixing Antibodies to the RS Virus in 1324 Sera Collected from August 1962 to July 1963*

As can be seen from Table 1 the sera were collected from most parts of Denmark. 661 of the sera were taken during the fall of 1962 and 663 sera during the spring of 1963. Moreover, the percentages concerning distribution according to locality were, broadly speaking, the same for the age-group 0-9 years as for the age-group 10-19 years. The total number of sera containing complement fixing antibodies to the RS virus was 171 (13 per cent). From Table 1 it is apparent also that positive sera can be obtained from every part of the country without accumulation of these in any particular area.

Fig. 1 shows the distribution of positive sera in relation to age. It is apparent that there is a steady increase in the numbers of positive reac-

TABLE 1

1324 Blood Specimens Examined for Complement Fixing Antibodies to the RS Virus

| Place of origin                                    | Numbers of sera examined | Numbers of LF positive sera |
|--|--------------------------|-----------------------------|
| Copenhagen   | 450 (33.7)*              | 54 (12.0)†                  |
| Zealand (excl. Copenhagen)                         | 152 (11.5)               | 20 (13.1)                   |
| The islands of Lolland and Falster                 | 34 (2.6)                 | 2 (5.8)                     |
| The island of Funen                                | 112 (8.5)                | 17 (15.2)                   |
| Northern Jutland (incl. the town of Aalborg)       | 112 (8.5)                | 21 (18.7)                   |
| The town of Aarhus                                 | 306 (23.2)               | 37 (12.0)                   |
| Central part of Jutland (excl. the town of Aarhus) | 121 (9.2)                | 15 (12.4)                   |
| Southern part of Jutland                           | 29 (2.2)                 | 3 (10.3)                    |
| The island of Bornholm                             | 8 (0.6)                  | 2 (25.0)                    |
| Total  | 1324                     | 171 (13.0)                  |

\* Per cent of total

† Per cent of the local number of sera

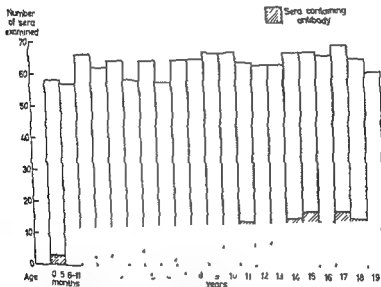


Fig. 1

Distribution according to age of 1324 sera examined with the RS virus antigen in Cx test during the period August 1962 to July 1963. The sera containing antibody are indicated.

tions the mean percentages for the age-groups, 0-4, 5-9, 10-14, and 15-19 being 5.2, 8.3, 16.9 and 23.2, respectively (the 58 sera from patients 0-5 months of age are not included). When the total numbers of positive sera from the two age groups 0-9 years and 10-19 years (42 and 129 respectively) are compared the difference is found statistically significant ( $p$  less than 0.001 in the chi square test). When the sera are

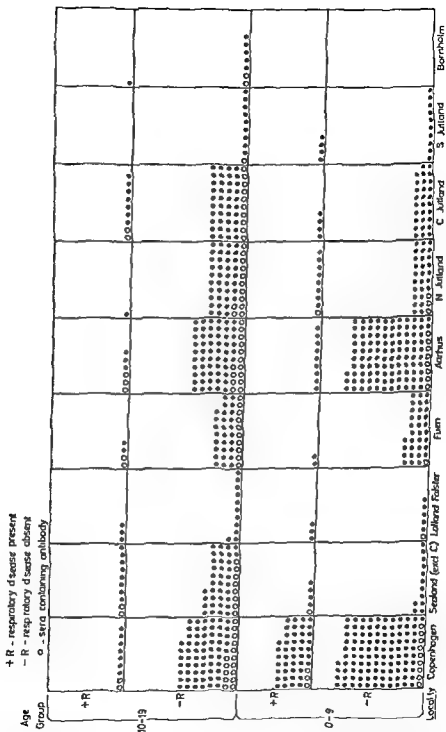


Fig 2

Distribution according to locality of sera from 705 patients without and 126 patients with acute respiratory disease at the time when the blood specimens were collected. The patients are divided into two age groups 0-9 and 10-19 years.

divided into two groups according to year of collection (August to December 1962 and February to July 1963) the same increase in the number of seropositives with age was seen for both these groups

A retrospective investigation of the 1324 sera involved was carried out. With the kind co-operation of hospitals and general practitioners it was possible to establish, with considerable certainty, from the case histories of 831 of the patients whether the patient had an acute respiratory disease at the time when the blood specimen was taken. Fig. 2 shows the result of the study of these patients. Statistically analysis of the data given in this scatter diagram indicates strongly that the presence of complement fixing antibodies to the RS virus had no relationship to the presence of acute respiratory diseases at the time the blood samples were taken neither in the younger nor in the older age groups. Moreover in accordance with Table 1 this figure demonstrates the fairly even geographical distribution of antibodies to the RS virus in individuals in this country.

The fact that the RS virus complement fixing antibodies are found so often in patients without acute respiratory diseases and the fact that the incidence of positive sera increases with age indicates strongly that these antibodies to the RS virus persist for many years in the blood of humans.

#### *Estimation of the RS Virus as an Aetiological Factor in Acute Respiratory Diseases in Children in Copenhagen in 1962-63*

101 paired sera from infants and children aged 0-9 years were examined for the presence of complement fixing antibodies to the RS virus. This group of sera originated from patients suffering from respiratory infections (Table 2) and represented most of the blood specimens sent to the Influenza Department from paediatric departments in Copenhagen hospitals from late September 1962 to the beginning of June 1963. The two specimens were taken at intervals of 5 to 13 days. The age distribution of the 101 patients is given in Fig. 3 which also shows the sera in which antibody to the RS virus was present. As can be seen, the antibodies were found in one or both serum samples in only 20 of these patients.

Table 3 shows the complement fixing antibody titres of the above-mentioned 20 pairs of sera showing the presence of antibodies, the corresponding age and clinical diagnosis of the patient together with the month in which the first blood sample was taken. It can be seen that eight pairs of sera gave a more than two-fold rise in titres. Moreover as many as seven of these were found among the 58 serum pairs taken in January, February and March whereas only one belonged to the 93 serum pairs collected in the months before and after this period. It is also apparent that all the patients who showed a significant rise in antibody titre were less than four years old and had pneumonic in

fections (Patient 13711 also had thrombocytopenic purpura and patient 13598 associated croup) None of the 20 patients had a CF antibody rise to influenza A, B or C, and only one (13115) had a CF antibody rise to adenovirus

TABLE 2  
*Distribution According to Main Diagnosis of 151 Infants and Children with Acute Respiratory Disease Admitted to Hospitals in Copenhagen from September 1962 to June 1963*

| Diagnosis                    | Number of patients |
|------------------------------|--------------------|
| Pneumonitis                  | 73                 |
| Bronchopneumonitis           | 17                 |
| Acute laryngitis             | 8                  |
| Primary atypical pneumonitis | 0                  |
| Viral pneumonitis            | 6                  |
| Acute bronchitis             | 22                 |
| Pneumonic infiltration       | 6                  |
| Acute rhinopharyngitis       | 6                  |
| Catarrhalia                  | 4                  |
| Total                        | 151                |

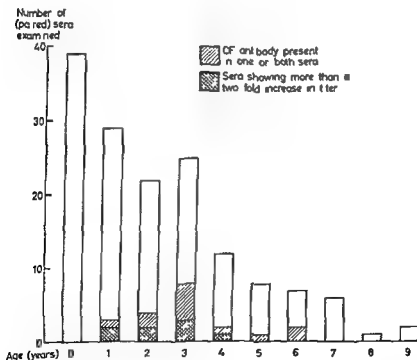


Fig 3

Distribution according to age of 151 paired sera from patients with acute respiratory disease in the Copenhagen area in the period September 1962-June 1963. All sera were examined for CF antibodies to the respiratory syncytial virus. Those showing presence of antibody are indicated.

TABLE 3

*Complement Fixing Antibody Titres against the RS Virus, Age of Patients, Clinical Diagnosis and Month in which First Blood Sample is Obtained from 20 Infants and Children with Acute Respiratory Disease in Copenhagen during the Period September 1962 to June 1963*

| Patient no | CF titre |      | Age of patient | Clinical diagnosis | Month in which first blood sample is obtained |
|------------|----------|------|----------------|--------------------|---|
|            | I        | II   |                |                    |   |
| 12920      | < 4      | 4    | 3              | Pneumonitis        | September 1962                                |
| 13115      | 16       | < 4  | 3              | "                  | October                                       |
| 13151      | 8        | 32   | 3              | "                  | "   |
| 13360      | 32       | 8    | 3              | Rhinopharyngitis   | December                                      |
| 13483      | < 64     | 32   | 1              | Pneumon infiltr    | January 1963                                  |
| 13551      | < 4      | > 64 | 2              | Pneumonitis        | "   |
| 13598      | 8        | > 64 | 4              | "                  | "   |
| 13603      | 8        | > 64 | 3              | "                  | February                                      |
| 13692      | < 4      | 8    | 1              | "                  | "   |
| 13711      | < 4      | 32   | 1              | "                  | "   |
| 13736      | 16       | 32   | 3              | "                  | "   |
| 13815      | > 64     | > 64 | 2              | "                  | March   |
| 13862      | < 4      | 32   | 3              | Pneumon infiltr    | "   |
| 13869      | < 4      | 8    | 2              | Pneumonitis        | "   |
| 14116      | 16       | 8    | 5              | "                  | April   |
| 14124      | 32       | 16   | 6              | Rhinopharyngitis   | "   |
| 14146      | 16       | 16   | 3              | Acute laryngitis   | "   |
| 14196      | 8        | 8    | 2              | Pneumonitis        | "   |
| 14223      | 32       | 16   | 4              | Acute bronchitis   | "   |
| 14247      | > 64     | 32   | 6              | Bronchopneumonitis | May   |

According to this data the RS virus seems to have played a part in the aetiology of the disease in question in 12 per cent of all the cases examined during January, February and March, 1963. From the other data in this part of our survey it is clearly seen that these 12 per cent represent an accumulation of cases, indicating that a wave of infections with this virus went through the community during the first three months of 1963.

#### *Incidence of CF Antibody to the Respiratory Syncytial Virus in Sera taken During Six Consecutive Years*

In order to estimate whether the incidence of antibodies differed particularly during successive years, 268 single sera from the years 1957-1962 were examined. They had all been found negative by the psittacosis complement fixation test, as is generally the case with the age-group concerned (0-9 years). Fig. 4 shows the distribution of the 268 sera examined according to the years during which they were taken. For each year the patients are divided into two age-groups, 0-4 and 5-9 years. The number of sera showing the presence of antibodies is also indicated. When the total numbers of seropositives from two successive years are compared by the chi square test, no statistically significant

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| Catarrhalia                  | 4                  |
| Total                        | 151                |

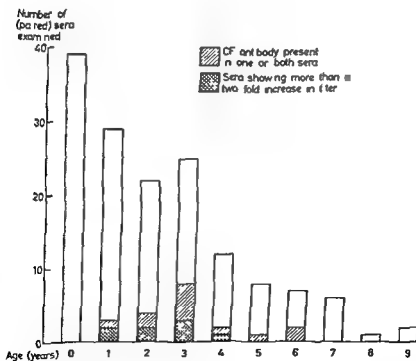


Fig 3

Distribution according to age of 151 paired sera from patients with acute respiratory disease in the Copenhagen area in the period September 1962-June 1963. All sera were examined for CF antibodies to the respiratory syncytial virus. Those showing presence of antibody are indicated.

TABLE 3

*Complement Fixing Antibody Titres against the RS Virus Age of Patients Clinical Diagnosis and Month in which First Blood Sample is Obtained from 20 Infants and Children with Acute Respiratory Disease in Copenhagen during the Period September 1962 to June 1963*

| Patient no | CF titre |      | Age of patient | Clinical diagnosis | Month in which first blood sample is obtained |
|------------|----------|------|----------------|--------------------|---|
|            | I        | II   |                |                    |   |
| 12920      | < 4      | 4    | 3              | Pneumonitis        | September 1962                                |
| 13115      | 16       | < 4  | 3              | "                  | October                                       |
| 13151      | 8        | ■    | 3              | "                  | "   |
| 13360      | 32       | 8    | 3              | Rhinopharyngitis   | December                                      |
| 13483      | < 64     | 32   | 1              | Pneumon infiltr    | January 1963                                  |
| 13551      | < 4      | > 64 | 2              | Pneumonitis        | "   |
| 13598      | 8        | > 64 | 4              | "                  | "   |
| 13653      | 8        | > 64 | 3              | "                  | February                                      |
| 13692      | < 4      | 8    | 1              | "                  | "   |
| 13711      | < 4      | 32   | 1              | "                  | "   |
| 13736      | 16       | 32   | 3              | "                  | "   |
| 13815      | > 64     | > 64 | 2              | "                  | March   |
| 13862      | < 4      | 32   | 3              | Pneumon infiltr    | "   |
| 13869      | < 4      | 8    | 2              | Pneumonitis        | "   |
| 14116      | 16       | 8    | 5              | "                  | April   |
| 14124      | 32       | 16   | 6              | Rhinopharyngitis   | "   |
| 14146      | 16       | 16   | 3              | Acute laryngitis   | "   |
| 14196      | 8        | 8    | 2              | Pneumonitis        | "   |
| 14223      | 32       | 16   | 4              | Acute bronchitis   | "   |
| 14247      | > 64     | 32   | 6              | Bronchopneumonitis | May   |

According to this data the RS virus seems to have played a part in the aetiology of the disease in question in 12 per cent of all the cases examined during January, February and March, 1963. From the other data in this part of our survey it is clearly seen that these 12 per cent represent an accumulation of cases, indicating that a wave of infections with this virus went through the community during the first three months of 1963.

#### *Incidence of CF Antibody to the Respiratory Syncytial Virus in Sera taken During Six Consecutive Years*

In order to estimate whether the incidence of antibodies differed particularly during successive years, 268 single sera from the years 1957-1962 were examined. They had all been found negative by the pertussis complement fixation test, as is generally the case with the age-group concerned (0-9 years). Fig. 4 shows the distribution of the 268 sera examined according to the years during which they were taken. For each year the patients are divided into two age-groups, 0-4 and 5-9 years. The number of sera showing the presence of antibody is indicated. The years are:



fections (Patient 13711 also had thrombocytopenic purpura and patient 13598 associated croup) None of the 20 patients had a CF antibody rise to influenza A, B or C, and only one (13115) had a CF antibody rise to adenovirus

TABLE 2  
*Distribution According to Main Diagnosis of 151 Infants and Children with Acute Respiratory Disease Admitted to Hospitals in Copenhagen from September 1962 to June 1963*

| Diagnosis                    | Number of patients |
|------------------------------|--------------------|
| Pneumonitis                  | 73                 |
| Bronchopneumonitis           | 17                 |
| Acute laryngitis             | 8                  |
| Primary atypical pneumonitis | 9                  |
| Viral pneumonitis            | 6                  |
| Acute bronchitis             | 22                 |
| Pneumonic infiltration       | 6                  |
| Acute rhinopharyngitis       | 6                  |
| Catarrhalia                  | 4                  |
| Total                        | 151                |

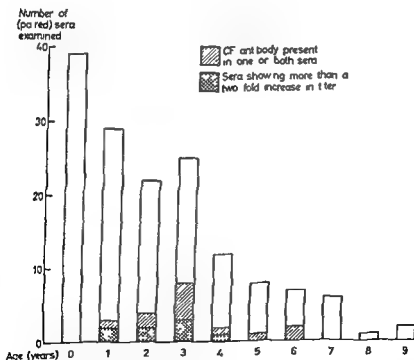


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| Patient no | CF titre |      | Age of patient | Clinical diagnosis | Month in which first blood sample was obtained |
|------------|----------|------|----------------|--------------------|--|
|            | I        | II   |                |                    |  |
| 12990      | < 4      | 4    | 3              | Pneumonitis        | September 1962                                 |
| 13115      | 16       | < 4  | 3              | "                  | October  |
| 13151      | 8        | 32   | 3              |                    | "  |
| 13360      | 32       | 8    | 3              | Rhinopharyngitis   | December                                       |
| 13483      | < 64     | 32   | 1              | Pneumon infiltr    | January 1963                                   |
| 13551      | < 4      | > 64 | 2              | Pneumonitis        |  |
| 13598      | 8        | > 64 | 4              |                    |  |
| 13623      | 8        | > 64 | 3              |                    | February                                       |
| 13692      | < 4      | 8    | 1              |                    |  |
| 13711      | < 4      | 32   | 1              |                    |  |
| 13738      | 16       | 32   | 3              |                    |  |
| 13815      | > 64     | > 64 | 2              |                    | March  |
| 13862      | < 4      | 32   | 3              | Pneumon infiltr    |  |
| 13869      | < 4      | 8    | 2              | Pneumonitis        |  |
| 14116      | 16       | 8    | 5              |                    | April  |
| 14124      | 32       | 16   | 6              | Rhinopharyngitis   | "  |
| 14146      | 16       | 16   | 3              | Acute laryngitis   |  |
| 14196      | 8        | 8    | 2              | Pneumonitis        | "  |
| 14223      | 32       | 16   | 4              | Acute bronchitis   |  |
| 14247      | > 64     | 32   | 6              | Bronchopneumonitis | May  |

According to this data the RS virus seems to have played a part in the aetiology of the disease in question in 12 per cent of all the cases examined during January February and March 1963. From the other data in this part of our survey it is clearly seen that these 12 per cent represent an accumulation of cases indicating that a wave of infections with this virus went through the community during the first three months of 1963.

#### *Incidence of CF Antibody to the Respiratory Syncytial Virus in Sera taken During Six Consecutive Years*

In order to estimate whether the incidence of antibodies differed particularly during successive years 268 single sera from the years 1957-1962 were examined. They had all been found negative by the psittacosis complement fixation test as is generally the case with the age group concerned (0-9 years). Fig. 4 shows the distribution of the 268 sera examined according to the years during which they were taken. For each year the patients are divided into two age groups 0-4 and 5-9 years. The number of sera showing the presence of antibody is indicated. The years are:

1959-1962 showed a slight, gradual decrease in the percentage of sero positives during those years

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(Head Arne Lithander, MD)

## CONCENTRATION OF PENICILLIN IN HUMAN PLASMA

### *Investigation of the Relation to the Content of Albumin and of Total Protein in Plasma*

By

ARNE LITHANDER

Received 27 IV 64

Chemotherapeutic preparations are known to be bound to blood plasma in varying degree. The extent to which this occurs differs from one preparation to another. It also differs for the plasma of different animal species. In the case of penicillin, research has revealed a considerable degree of binding to plasma and wide variations between different kinds of penicillin. Klein (1), for example, stated that the activity of penicillin in human plasma was only 50 per cent of that in buffer solution. Tompsett *et al* (2) reported that penicillin K lost 85 to 90 per cent of its activity in 30 per cent human serum, while penicillin G lost 40 to 60 per cent of its activity when similarly tested. Smith *et al* (3) found that the degree of binding of penicillin V by human plasma was significantly higher than that of penicillin G. Chow & McKee (4) showed that penicillin was bound by human albumin in water solution at the concentration present in normal human plasma. Lithander (5) reported that benzyl penicillin activity was relatively more reduced by rabbit plasma when the penicillin concentration was low than when it was high.

The following investigation was conducted to determine in what way the binding of penicillin is dependent on the concentration in human plasma of, for example, albumin.

### MATERIALS AND METHODS

Samples of venous blood were taken from 30 patients and mixed with a small amount of heparin. None of the patients had been given antibacterial preparations. Just prior to the removal of these samples an electrophoretic investigation was made of the concentration of total protein, albumin and the different globulin fractions in plasma. Immediately after the samples were taken the plasma was separated from the corpuscles by centrifugation. Benzyl penicillin<sup>1</sup> was dissolved in plasma or dilutions of plasma so as to secure different concentrations. The penicillin con-

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<sup>1</sup>The penicillin in the present investigation was kindly supplied by AB Kabi Stockholm Sweden.

Hereinafter the term benzyl penicillin will be abbreviated to penicillin.

centrations were 0.5, 0.25, 0.125 and 0.0625 IU per ml. The plasma was used both undiluted and diluted with phosphate buffer solutions 1/2, 1/5, 1/10 and 1/20.

Diffusion technique in agar was used to determine the penicillin content of the abovementioned solutions of penicillin. *Subtilis* spores of the ATCC 6633 strain were used as test organisms. The spores were suspended in melted agar in a concentration of 7 500 spores per ml of agar. The agar was poured into Petri dishes. When it had solidified, seven cylindrical holes were punched out on each plate. Then 0.06 ml of phosphate buffer solutions of standard penicillin containing 0.12, 0.06 and 0.03 IU per ml, respectively, were introduced into three of the holes in each plate. The remaining holes were filled with 0.06 ml of the penicillin solutions in plasma or dilutions thereof to be tested. Five plates were used in each test. Readings were made following incubation at 37° C for 18 hours. Two diameters at right angles to the

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## RESULTS

In Table 1 we find the concentrations of albumin and total protein in plasma. Table 2 shows the concentrations of albumin and total protein in plasma. Table 3 also contains the correlations between the concentration of unbound penicillin and the content of the total protein of the plasma.

TABLE 1  
Concentration of Albumin and Total Protein in Plasma  
Means Are Expressed in gm/100 ml  
Concentration in plasma

|               | n  | $\bar{x} \pm \sigma_{\bar{x}}$ | $\sigma_{\bar{x}}$ | v    |
|---------------|----|--------------------------------|--------------------|------|
| Albumin       | 30 | $3.7 \pm 0.22$                 | 1.22               | 33.0 |
| Total protein | 30 | $7.3 \pm 0.20$                 | 1.09               | 15.0 |

The concentrations of albumin varied between 1.44 and 6.71 and those of total protein between 4.50 and 9.78 gm/100 ml.

It appears from Table 2 and Fig. 1 that there was a

greater the lower the concentration of penicillin administered. When

TABLE 2

*Yield of Unbound Penicillin in Different Dilutions of Human Plasma  
Yield Is Expressed in per Cent of the Concentration of the Penicillin Dose*

| Concentration of penicillin dosage in plasma IU per ml | Dilution of plasma | n  | Yield of unbound penicillin in plasma |            |      |         |
|--|--------------------|----|---------------------------------------|------------|------|---------|
|  |                    |    | $\bar{x} \pm \sigma_x$                | $\sigma_x$ | $v$  | $t$     |
| 0.5  | Undiluted          | 30 | $80.2 \pm 1.54$                       | 8.42       | 10.5 | -12.853 |
|  | 1/2.5              | 30 | $87.8 \pm 1.53$                       | 8.36       | 9.5  | -7.973  |
|  | 1/5                | 30 | $92.0 \pm 1.47$                       | 8.04       | 8.7  | -5.457  |
|  | 1/10               | 30 | $94.8 \pm 1.33$                       | 7.30       | 7.7  | -3.849  |
|  | 1/20               | 30 | $98.3 \pm 1.03$                       | 5.89       | 6.0  | -1.544  |
| 0.25   | Undiluted          | 30 | $74.3 \pm 1.50$                       | 8.24       | 11.1 | -17.095 |
|  | 1/2.5              | 30 | $82.4 \pm 1.38$                       | 7.54       | 9.2  | -12.777 |
|  | 1/5                | 30 | $86.9 \pm 1.23$                       | 6.73       | 7.8  | -10.693 |
|  | 1/10               | 30 | $89.9 \pm 1.04$                       | 5.71       | 6.4  | -9.688  |
|  | 1/20               | 30 | $93.1 \pm 0.81$                       | 4.43       | 4.8  | -8.531  |
| 0.125  | Undiluted          | 30 | $71.6 \pm 1.30$                       | 7.10       | 10.0 | -21.878 |
|  | 1/2.5              | 30 | $80.5 \pm 1.03$                       | 5.66       | 7.0  | -18.889 |
|  | 1/5                | 30 | $86.8 \pm 0.92$                       | 5.06       | 5.8  | -14.244 |
|  | 1/10               | 30 | $90.1 \pm 0.90$                       | 4.93       | 5.5  | -10.965 |
|  | 1/20               | 30 | $91.6 \pm 0.72$                       | 3.93       | 4.3  | -11.721 |
| 0.0625   | Undiluted          | 30 | $61.1 \pm 5.88$                       | 32.2       | 52.7 | -6.623  |
|  | 1/2.5              | 30 | $75.9 \pm 1.47$                       | 8.03       | 10.6 | -16.459 |
|  | 1/5                | 30 | $84.1 \pm 1.16$                       | 6.33       | 7.5  | -13.775 |
|  | 1/10               | 30 | $88.9 \pm 0.81$                       | 4.45       | 5.0  | -13.649 |
|  | 1/20               | 30 | $92.9 \pm 0.63$                       | 3.45       | 3.7  | -11.272 |

the concentration of penicillin was 0.5 IU per ml, the plasma had no effect in a dilution of 1/20. With lower concentrations of penicillin, plasma in a dilution of 1/20 did have a significant effect.

Table 3 reveals that the binding of penicillin was significant, depending on the concentration of albumin and protein in plasma. The correlation to the concentration of albumin was more pronounced than to the concentration of total protein. The yield in per cent of unbound penicillin decreased with increasing albumin content. The penicillin yield in undiluted plasma decreased by an average of 4 per cent with an increase in albumin content of 1 per cent. Fig. 2 illustrates that the yield of penicillin with different calculated penicillin concentrations depended on the concentration of albumin in undiluted plasma. The slope of the regression lines is about the same for the four penicillin dosages used.

Comparisons between the results of penicillin determinations in plasma or dilutions thereof and the concentration of the various globulin fractions in plasma revealed no significant relation between the concentration of unbound penicillin, on the one hand, and the concentration of any globulin fraction on the other.

Yield of unbound penicillin in per cent

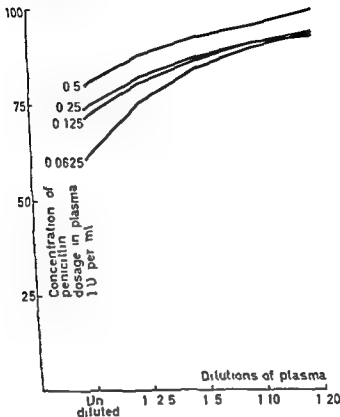


Fig 1

Yield of unbound penicillin in different dilutions of human plasma. Yield is expressed in per cent of the concentration of the penicillin administered. The results (means) are the same as in Table 2.

### COMMENTS

In the treatment of infections with penicillin it is sometimes helpful to determine in advance the concentration of free penicillin which will be found in the patient's blood plasma. The dosage of penicillin is adapted to suit the nature of the infection and in localized infections, the site of the infection. Consequently it may be of interest to know whether the binding to plasma alters when the dosage of penicillin is varied.

Several researchers have shown that penicillin is bound to plasma and also that albumin in concentrations corresponding to the concentration in normal human plasma binds penicillin.

The present investigations revealed that the concentration of albumin



TABLE 2

*Yield of Unbound Penicillin in Different Dilutions of Human Plasma  
Yield Is Expressed in per Cent of the Concentration of the Penicillin Dose*

| Concentration of penicillin dosage in plasma IU per ml | Dilution of plasma | n  | Yield of unbound penicillin in plasma |          |      |         |
|--|--------------------|----|---------------------------------------|----------|------|---------|
|  |                    |    | $\bar{x} \pm \sigma$                  | $\sigma$ | v    | t       |
| 0.5  | Undiluted          | 30 | 80.2 $\pm$ 1.54                       | 8.42     | 10.5 | -12.853 |
|  | 1/2.5              | 30 | 87.8 $\pm$ 1.53                       | 8.36     | 9.5  | -7.973  |
|  | 1/5                | 30 | 92.0 $\pm$ 1.47                       | 8.04     | 8.7  | -5.457  |
|  | 1/10               | 30 | 94.8 $\pm$ 1.33                       | 7.30     | 7.7  | -3.879  |
|  | 1/20               | 30 | 98.3 $\pm$ 1.08                       | 5.89     | 6.0  | -1.544  |
| 0.25   | Undiluted          | 30 | 74.3 $\pm$ 1.50                       | 8.24     | 11.1 | -17.095 |
|  | 1/2.5              | 30 | 82.4 $\pm$ 1.38                       | 7.54     | 9.2  | -12.777 |
|  | 1/5                | 30 | 86.9 $\pm$ 1.23                       | 6.73     | 7.8  | -10.693 |
|  | 1/10               | 30 | 89.9 $\pm$ 1.04                       | 5.71     | 6.4  | -9.688  |
|  | 1/20               | 30 | 93.1 $\pm$ 0.81                       | 4.43     | 4.8  | -8.531  |
| 0.125  | Undiluted          | 30 | 71.6 $\pm$ 1.30                       | 7.10     | 10.0 | -21.878 |
|  | 1/2.5              | 30 | 80.5 $\pm$ 1.07                       | 5.66     | 7.0  | -18.889 |
|  | 1/5                | 30 | 86.8 $\pm$ 0.92                       | 5.06     | 5.8  | -14.244 |
|  | 1/10               | 30 | 90.1 $\pm$ 0.90                       | 4.93     | 5.5  | -10.965 |
|  | 1/20               | 30 | 91.6 $\pm$ 0.72                       | 3.93     | 4.3  | -11.721 |
| 0.0625   | Undiluted          | 30 | 61.1 $\pm$ 5.88                       | 32.2     | 52.7 | -6.623  |
|  | 1/2.5              | 30 | 75.9 $\pm$ 1.47                       | 8.03     | 10.6 | -16.459 |
|  | 1/5                | 30 | 84.1 $\pm$ 1.16                       | 6.33     | 7.5  | -13.775 |
|  | 1/10               | 30 | 88.9 $\pm$ 0.81                       | 4.45     | 5.0  | -13.639 |
|  | 1/20               | 30 | 92.9 $\pm$ 0.63                       | 3.45     | 3.7  | -11.272 |

the concentration of penicillin was 0.5 IU per ml, the plasma had no effect in a dilution of 1/20. With lower concentrations of penicillin, plasma in a dilution of 1/20 did have a significant effect.

Table 3 reveals that the binding of penicillin was significant, depending on the concentration of albumin and protein in plasma. The correlation to the concentration of albumin was more pronounced than to the concentration of total protein. The yield in per cent of unbound penicillin decreased with increasing albumin content. The penicillin yield in undiluted plasma decreased by an average of 4 per cent with an increase in albumin content of 1 per cent. Fig. 2 illustrates that the yield of penicillin with different calculated penicillin concentrations depended on the concentration of albumin in undiluted plasma. The slope of the regression lines is about the same for the four penicillin dosages used.

Comparisons between the results of penicillin determinations in plasma or dilutions thereof and the concentration of the various globulin fractions in plasma revealed no significant relation between the concentration of unbound penicillin, on the one hand, and the concentration of any globulin fraction on the other.

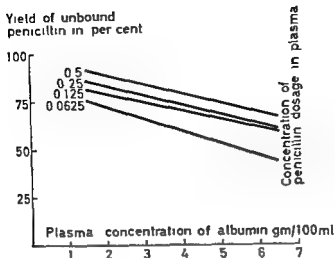


Fig 2

Relations between the concentration of unbound penicillin and the content of albumin in undiluted plasma

and total protein played an important part in the binding of penicillin when the dosage of penicillin varied between 0.06 and 0.5 IU per ml of plasma or dilutions thereof. The degree of binding varied with the penicillin level in plasma. The capacity to bind penicillin was diminished when the plasma was diluted, but it was appreciable even in relatively highly diluted plasma when the level of penicillin was low.

### SUMMARY

1 The binding of benzyl penicillin to human plasma was analysed with respect to the importance of the concentration of albumin and total protein.

2 The binding of benzyl penicillin was related to the concentration of albumin and total protein. The correlation to albumin was more pronounced than to total protein. This binding capacity of human plasma could be demonstrated up to a plasma dilution of 1/20.

3 In undiluted plasma the concentrations of unbound penicillin decreased on an average of 4 per cent for each 1 per cent increase in the albumin content.

### REFERENCES

1 Kleinfelder

2 Tomp

TABLE 3

*Correlations between the Concentration of Unbound Penicillin and the Concentration of Plasma Albumin and of Total Protein with Different Doses of Penicillin and Different Dilutions of the Plasma*

| Concentration of penicillin dosage in plasma (U per ml) | Dilutions of plasma | Correlations       |                    |                    |                    |
|---|---------------------|--------------------|--------------------|--------------------|--------------------|
|   |                     | Albumin            |                    | Total protein      |                    |
|   |                     | $b \pm \epsilon b$ | $r \pm \epsilon r$ | $b \pm \epsilon b$ | $r \pm \epsilon r$ |
| 0.5   | Undiluted           | $4.92 \pm 0.910$   | $0.71 \pm 0.089$   | $-2.88 \pm 1.350$  | $-0.37 \pm 0.157$  |
|   | 1/2.5               | $-4.13 \pm 1.030$  | $-0.61 \pm 0.115$  | $-2.21 \pm 1.390$  | $-0.29 \pm 0.167$  |
|   | 1/5                 | $3.74 \pm 1.015$   | $-0.57 \pm 0.123$  | $-2.36 \pm 1.310$  | $-0.32 \pm 0.164$  |
|   | 1/10                | $3.40 \pm 0.926$   | $-0.57 \pm 0.123$  | $-2.07 \pm 1.200$  | $-0.31 \pm 0.165$  |
|   | 1/20                | $2.27 \pm 0.802$   | $-0.47 \pm 0.142$  | $-2.56 \pm 0.898$  | $-0.47 \pm 0.142$  |
| 0.25  | Undiluted           | $-5.14 \pm 0.823$  | $-0.76 \pm 0.076$  | $-4.15 \pm 1.190$  | $-0.55 \pm 0.126$  |
|   | 1/2.5               | $3.92 \pm 0.899$   | $-0.64 \pm 0.109$  | $-3.61 \pm 1.120$  | $-0.52 \pm 0.133$  |
|   | 1/5                 | $3.07 \pm 0.862$   | $-0.56 \pm 0.126$  | $-3.07 \pm 1.001$  | $-0.50 \pm 0.137$  |
|   | 1/10                | $-2.53 \pm 0.741$  | $-0.54 \pm 0.129$  | $-2.84 \pm 0.830$  | $-0.54 \pm 0.129$  |
|   | 1/20                | $-1.24 \pm 0.642$  | $-0.34 \pm 0.161$  | $-1.85 \pm 0.682$  | $-0.40 \pm 0.145$  |
| 0.125   | Undiluted           | $4.74 \pm 0.631$   | $-0.82 \pm 0.061$  | $-3.28 \pm 1.060$  | $-0.50 \pm 0.136$  |
|   | 1/2.5               | $2.59 \pm 0.723$   | $-0.61 \pm 0.115$  | $-2.69 \pm 0.837$  | $-0.52 \pm 0.132$  |
|   | 1/5                 | $2.03 \pm 0.680$   | $-0.49 \pm 0.138$  | $-2.44 \pm 0.745$  | $-0.53 \pm 0.132$  |
|   | 1/10                | $1.74 \pm 0.686$   | $-0.43 \pm 0.148$  | $-1.84 \pm 0.779$  | $-0.41 \pm 0.152$  |
|   | 1/20                | $1.55 \pm 0.531$   | $-0.49 \pm 0.140$  | $-1.08 \pm 0.649$  | $-0.30 \pm 0.166$  |
| 0.0625  | Undiluted           | $6.53 \pm 0.973$   | $-0.79 \pm 0.070$  | $-4.77 \pm 1.510$  | $-0.51 \pm 0.135$  |
|   | 1/2.5               | $-4.56 \pm 0.904$  | $-0.69 \pm 0.096$  | $-3.58 \pm 1.230$  | $-0.48 \pm 0.140$  |
|   | 1/5                 | $-2.83 \pm 0.817$  | $-0.57 \pm 0.128$  | $-2.72 \pm 0.986$  | $-0.47 \pm 0.142$  |
|   | 1/10                | $-1.54 \pm 0.622$  | $-0.42 \pm 0.150$  | $-0.94 \pm 0.749$  | $-0.23 \pm 0.173$  |
|   | 1/20                | $-1.07 \pm 0.493$  | $-0.38 \pm 0.157$  | $1.04 \pm 0.564$   | $-0.33 \pm 0.173$  |

$b$  = regression coefficient

$r$  = correlation coefficient

The International Escherichia Centre Statens Seruminstitut Copenhagen Denmark  
Central Veterinary Laboratory Weybridge England and  
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## K ANTIGENS K88ab(L) AND K88ac(L) IN *E coli*

*A New O Antigen 0147 and a New K Antigen K89(B)*

By

IDA ØRSKOV FRITS ØRSKOV W J SOJKA and W WITTIG

Received 8 v 64

In a previous paper (10) the existence of *E coli* strains with two kinds of k antigens a B antigen and an L antigen was reported. A description was given of two strains G7 and Γ68 isolated from swine which possessed different O B and H antigens but the same L antigen named k88. It was shown that the detection of this L antigen was simplified by the fact that it was not developed after growth at 18° C.

At first the k88 antigen was thought of as an entity as the two strains mentioned above had identical L antigens. Since then additional types with the k88 antigen were encountered among strains isolated from piglets in different countries. Investigation of such strains seemed to indicate that the k88 antigen was composed of several antigenic factors which could be present in different combinations. The present report deals with the result of these investigations.

### MATERIAL AND METHODS

#### Strains

The strains

4181 our strains above the line were isolated during the years 1960-63. G1253 G203 and G491 originated from England and were first analysed by Sojka while strain C81579 came from Germany and was first examined by Wittig in Dresden. A strain (C662 63) of the 0138 k81 type with the K88(L) antigen was received from Dr Wittig before G491 but was not investigated further.

#### Preparation of Ant sera

These were prepared by injecting rabbits five times at intervals of two or four to five days. In the first case the doses ranged from 0.1 to 1.0 ml in the second from 0.2 to 2.0 ml. The animals were bled seven or eight days after the last injection.

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TABLE 2  
Results of Slide Agglutination Test

| Antigens | Growth temp<br>°C | Sera                   |                   |          |                |
|----------|-------------------|------------------------|-------------------|----------|----------------|
|          |                   | Ok E68 — test h 88 (L) |                   | Ok G1253 |                |
|          |                   | Unabs                  | Abs by live G1253 | Unabs    | Abs by live G7 |
| G7       | 37                | +++                    | +++               | +++      | —              |
| G7       | 18                | —                      | —                 | —        | —              |
| G1253    | 37                | +++                    | —                 | +++      | +++            |
| G1253    | 18                | —                      | —                 | +++      | +++            |

The recording of a positive reaction as +++ means that the clumping is heavy

TABLE 3  
Results of Tube Agglutination

| Antigen | Cultiv temp<br>°C | Sera          |          |                |  |         |
|---------|-------------------|---------------|----------|----------------|--|---------|
|         |                   | Test h 88 (L) | Ok G1253 |                |  | O G1253 |
|         |                   |               | Unabs    | Abs by live G7 | Abs by homol 100 C cult = pure L serum |         |
| G7      | 37                | 1280          | 320      | 0              | 320                                    | 0       |
| G7      | 18                | 0             | 0        | —              | —                                      | —       |
| G1253   | 37*               | 160           | 640      | 160            | 320                                    | 80      |
| G1253   | 18                | 0             | 320      | 160            | 0                                      | 40      |
| G205    | 37*               | 320           | 320      | 640            | 320                                    | 0       |
| G205    | 18                | 0             | 0        | —              | 0                                      | —       |
| G491    | 37                | 160           | 320      | 320            | 320                                    | 0       |
| G491    | 18                | 0             | 0        | —              | 0                                      | —       |

\* These cultures agglutinated to the same extent in the saline control tube, revealed by means of hand lens only  
Titre is the reciprocal value of the highest serum dilution showing clumping visible to the naked eye

strable if the culture has been grown at 18° C. This fact is indicative of the presence of K88 antibodies in OK serum G1253. Similarly, only the 37° C culture of G1253 reacts in serum h 88, which points to the same fact, that is that the I antigen K88 is present in this strain. Since G1253 is incapable of removing the antibodies from K88 serum reacting with culture G7, the K88 antigen of the two strains cannot be identical. From the table it is not evident whether G7 can absorb the K88 antibodies from the OK serum G1253, as culture G1253 agglutinates in the absorbed serum regardless of cultivation temperature. As this reaction might be a B (or an O) antigen reaction, the serum has to be depleted of B and O antibodies by saturation with the homologous boiled culture before absorption with live culture of G7. Before showing the results of such absorptions in Table 4, we show the results of some tube agglutination tests in Table 3, strains G205 and G491 are

TABLE 1  
*Strains Used*

| Strain no | Antigens |                   |                   |    | Hae molysis | Received from                 |
|-----------|----------|-------------------|-------------------|----|-------------|-------------------------------|
|           | O        | B                 | K I               | H  |             |                               |
| G7        | 8        | 87                | 8 <sup>9</sup> ab | 19 | +           | Sojka Weybridge England       |
| E68       | 141      | 8 <sup>5</sup> ab | 88ab              | 4  | +           | '                             |
| G1253     | 147      | 89                | 88ac              | 19 | +           | '                             |
| G205      | 8        | 87                | 88ac              | 19 | +           | '                             |
| G491      | 138      | 81                | 88ac              | 19 | —           | '                             |
| CS1522    | (1117)   | ne                | 88 <sup>1</sup> c | 10 | —           | Wittig Dresden Germany        |
| G1108F    | 141      | 8 <sup>5</sup> ac | 88ab              | 4  | +           | Sojka Weybridge, England      |
| (258 61)  | 147      | ne                | 88ac              | 19 | +           | Thal Stockholm, Sweden        |
| C794 61   | 147      | ne                | 88ac              | 19 | +           | Sorum Oslo, Norway            |
| (789 61)  | 8        | 87                | 88ac              | 19 | +           | '                             |
| C105 62   | 147      | ne                | 88ac              | 19 | +           | Wittig Dresden Germany        |
| C462-62   | 147      | ne                | 88ac              | 19 | +           | '                             |
| (467-62)  | 8        | 87                | 88 <sup>1</sup> c | *? | +           | '                             |
| C308 63   | unknown  | ne                | 88a?              | 43 | —           | '                             |
| C662 63   | 138      | 81                | 88ac              | 19 | +           | '                             |
| C667-63   | (115)    | ne                | 88a?              | 10 | —           | '                             |
| C1160 63  | 8        | 87                | 88ac              | 19 | +           | Djurisic, Novi Sad Yugoslavia |

\*? means not motile enough for H determination

ne — not examined

Only K88 factors in italic have been fully verified experimentally

## RESULTS

*The K Antigen of the L Type*

The three new English strains included in this study, all of different O antigen type, had one particular property in common they very often agglutinated in saline when tested as live culture grown at 37° C, but the agglutination varied in extent from time to time. After heating to 100° C for one hour the autoagglutinability disappeared, and it was never present in live cultures grown at 18° C. Because of the instability in saline the results of the agglutination were difficult to interpret in some cases, but in others it was thought justifiable to rely upon an agglutination in serum if the intensity of this was unquestionably greater than that of the control. Slide agglutination was generally more useful than tube agglutination.

O and OK sera produced from strain G1253 were used throughout the study. In addition OK sera prepared with the previously described G7 and E68 strains were employed. As will be remembered, these two strains have identical K88(L) antigens. As one of the cultures studied, G205, had O, B and H antigens similar to G7, only the reactions in the E68 OK serum are presented here.

In Table 2 it is shown that G7 agglutinates in the OK serum G1253 in the same manner as in the OK serum E68, designated here and in the following tables as test K88(I), i.e. no agglutination is demon-

TABLE 2  
Results of Slide Agglutination Test

| Antigens | Growth temp °C | Sera                  |                   |          |                |
|----------|----------------|-----------------------|-------------------|----------|----------------|
|          |                | Ok 168 - test k88 (L) |                   | Ok G1253 |                |
|          |                | Unabs                 | Abs by live G1253 | Unabs    | Abs by live G7 |
| G7       | 37             | +++                   | +++               | +++      | —              |
| G7       | 18             | —                     | —                 | —        | —              |
| G1253    | 37             | +++                   | —                 | +++      | +++            |
| G1253    | 18             | —                     | —                 | +++      | +++            |

The recording of a positive reaction as +++ means that the clumping is heavy

TABLE 3  
Results of Tube Agglutination

| Antigen | Cultiv temp °C | Sera          |          |                |  |         |
|---------|----------------|---------------|----------|----------------|--|---------|
|         |                | Test k 88 (L) | Ok G1253 |                |  | O G1253 |
|         |                |               | Unabs    | Abs by live G7 | Abs by homol 100 C cult = pure L serum |         |
| G7      | 37             | 1280          | 320      | —              | 320                                    | 0       |
| G7      | 18             | 0             | 0        | —              | —                                      | —       |
| G1253   | 37*            | 160           | 640      | 160            | 320                                    | 80      |
| G1253   | 18             | 0             | 320      | 160            | 0                                      | 40      |
| G205    | 37*            | 320           | 320      | 640            | 320                                    | 0       |
| G205    | 18             | 0             | 0        | —              | —                                      | —       |
| G491    | 37             | 160           | 320      | 320            | 320                                    | 0       |
| G491    | 18             | 0             | 0        | —              | 0                                      | —       |

\* These cultures agglutinated to the same extent in the saline control tube revealed by means of hand lens only  
Titre is the reciprocal value of the highest serum dilution showing clumping visible to the naked eye

strable if the culture has been grown at 18° C. This fact is indicative of the presence of K88 antibodies in OK serum G1253. Similarly, only the 37° C culture of G1253 reacts in serum K88, which points to the same fact that is that the L antigen K88 is present in this strain. Since G1253 is incapable of removing the antibodies from K88 serum reacting with culture G7, the K88 antigen of the two strains cannot be identical. From the table it is not evident whether G7 can absorb the K88 antibodies from the OK serum G1253, as culture G1253 agglutinates in the absorbed serum regardless of cultivation temperature. As this reaction might be a B (or an O) antigen reaction, the serum has to be depleted of B and O antibodies by saturation with the homologous boiled culture before absorption with live culture of G7. Before showing the results of such absorptions in Table 4, we show the results of some tube agglutination tests in Table 3, strains G205 and G491 are



included. From this it appears that strain G1253 has an L antigen, as was to be anticipated from Table 2, since absorption by homologous boiled culture leaves antibodies reacting with the live 37° C culture. The 18° C culture is negative in this pure L serum, just as it is negative in the test K88 serum. In addition, the table shows that strains G205 and G491 also have a K88 antigen complex, since 37° C cultures agglutinate both in the pure L serum of G1253 and in the test K88 serum. Since they also agglutinate in OK serum G1253 after absorption of this with G7, their K88 antigen is not identical with that of G7.

TABLE 4  
*Results of Slide Agglutination Tests*

| Anti-<br>gens | Sera        |                     |      |        |                      |                             |      |        |
|---------------|-------------|---------------------|------|--------|----------------------|-----------------------------|------|--------|
|               | Test K88(I) |                     |      |        |                      | Pure L of G1253             |      |        |
|               | Unabs       | Abs by live cult of |      |        | No<br>further<br>abs | Further abs by live cult of |      |        |
|               |             | G1253               | G205 | CS1522 |                      | G7                          | G205 | CS1522 |
| G7            | +++         | +++                 | +++  | +++    | +++                  | —                           | —    | +      |
| G1253         | +++         | —                   | —    | +      | +++                  | +++                         | —    | +      |
| G205*         | +++         | —                   | —    | +      | +++                  | +++                         | —    | +      |
| G491†         | +++         | ?                   | ?    | ?      | +++                  | +++                         | ?    | ?      |
| CS1522        | +++         | —                   | —    | —      | +++                  | +++                         | —    | —      |

All cultures were grown at 37° C

18° C cultures of all strains including CS1522 reacted as shown in the preceding tables, and are therefore omitted

- \* This culture showed clumping in saline which was visible only with the aid of a hand lens  
 † The clumping in saline of this culture was visible to the naked eye  
 +++ versus + means difference in degree of agglutination

Additional absorptions of OK serum G1253 absorbed by homologous 100° C culture, *i.e.* the pure L serum, were thereafter carried out. In three separate experiments live cultures of G7, G205 and CS1522 were used as absorbing agents. Strain CS1522 was included in the study as it was not autoagglutinable and probably had a K88 antigen resembling that of G1253. The test K88 serum was absorbed by the same cultures. The results of slide agglutination tests performed with these absorbed sera are shown in Table 4. From this it is evident that the K88(L) antigens of G7 and G1253 have an ab, ac relationship. This is deduced from the information, also presented in Table 2 regarding the absorption of the test K88 serum by culture G1253, and from the fact, as shown in Table 4, that G7 is incapable of removing the L antibodies from the pure L serum of G1253. The K88 antigen factors of the strains in question can then be expressed in the following way

|               |        |
|---------------|--------|
| K88a b(L)     | F68    |
| K88a b(L)     | G7     |
| K88a c(L)     | G1253  |
| K88a c ?(L)   | G205   |
| K88(a) c ?(L) | CS1522 |

As no sera of strains G205 and CS1522 were examined in this study, nothing is known about additional K88 factors of these strains. This is expressed by a question-mark (?) in the formulas. Strain CS1522 seems to have a K88 antigen which closely resembles that of G1253, but the absorptions indicate that the a factor is not complete. For this reason it is placed in brackets. No formula is given for G491, as this culture was not used as absorbing culture, and in the experiments presented in Table 4 it was autoagglutinable. In Table 1 the K88 antigen of G491 is given as K88ac.

Following the finding of the K88ac(L) antigen factors in both strain G1253 (0147) and strain G205 (08), 53 strains considered to be, and labelled as, "G7" types were re-examined in Weybridge by one of the authors. By slide agglutination technique using OK serum of G1253 absorbed with the G7 strain, it was shown that only 43 per cent of the strains were of the "G7" type, i.e. they had the K88ab factors, while the remaining strains were of the "G205" type. In Table 1 a list is given of strains with K88(L) antigen received in the International Escherichia Centre. Each strain is a representative of several strains from the same laboratory.

In the G7 strain itself some sort of an antigenic variation from the b to the a factor of K88 has been looked for, at one time 670 single colonies originating from one colony were checked in absorbed sera, but none was found to have the ac factors.

As stated previously (9), strain G7 is a genetic donor, i.e. when G7 is seeded with another coli strain the latter acquires the K88(L) antigen and the recipient strain can furthermore now act as donor in genetic crosses. Up to the present it has not been possible to demonstrate the transfer of the K88ac(L) antigen from either G1253 or G205 to a recipient (H509a), which in a parallel experiment was capable of receiving the K88ab(L) antigen from G7.

### Other Antigens

For information of the other antigens possessed by the strains, the reader is referred to the sero formulas given in Table 1. The determination of these antigens was carried out by standard procedures. When the O, H and K antigens are assigned to known types this does not mean that the identity with antigens of test types has been demonstrated by serum production and cross absorption, but that agglutination tests have showed reactions which justified the serotypes given.

One new O antigen (0147) was established. This antigen has some relationship to the O groups already established, particularly to 0133,

013, 0139 and 0102. At first it was not considered desirable to let strain G1253 represent a new O group, as it might be regarded as belonging to O group 133. However, many strains of this group, isolated from diseased swine in different countries, were received in the International Escherichia Centre, and it thus seemed to be a common swine strain. In some cases the strains agglutinated only at low titre in the anti 0133 serum. This means that the reaction might be overlooked. Moreover, it was shown in reciprocal cross absorption tests that strain G1253 and test strain for O antigen 133 had special O antigen factors. For these reasons it was decided to establish 0147 as a new O antigen.

As the L antigen of culture G1253 is suppressed by 18° C cultivation, inagglutinability in O serum of an 18° C culture is presumably caused by the presence of a K antigen of the B type. The 18° C culture agglutinated well in the OK serum and could, after boiling, remove all agglutinins reacting with the 18° C culture (see Table 5). This means that the strain has a B antigen. This B antigen was numbered K89(B), as it was not found to be related to any of the K antigens established previously, except that the test strain of K21 agglutinated to some extent in the OK serum G1253. The reverse test was negative.

TABLE 5  
*Demonstration of a B Antigen at G1253*

| Antigens                     | Sera    |          |                          |
|------------------------------|---------|----------|--------------------------|
|                              | O G1253 | OK G1253 |                          |
|                              |         | Unboiled | Abx by boiled G1253 18°C |
| Formalinized G1253 18° C     | 20      | 160      | 0                        |
| Heated to 100° C G1253 18° C | 1280    | 1280     | 11                       |

The O antigen of strain CS1522 has been designated (01,0117), which means that it has some relationship to both these O groups. A number of strains of this type have been received during the last two or three months, all from one laboratory (Dr Wittig, Dresden). Future findings will show whether also this type deserves its own O antigen number. Strain CS1522 was not examined for B antigen.

The H antigens especially represented among the strains are H4, H19 and H10. It is noteworthy that the 08, the 0148 and the 0147 strains all had H antigen 19. Common to strains with the K88(L) antigen and the H19 antigen is the fact that they are poorly motile. For this reason the H determination can be time-consuming and at first apparently unfeasible. H10 was found in two strains of different O groups (see Table 1).

At this juncture it would be appropriate to mention the peculiar fact that the patterns of the fermentation reactions seem to follow the H

antigens (Table 6) Three different patterns were found corresponding to the three different H antigens. Even those strains mentioned above, which had H10 but different O antigens, had the same fermentation pattern. It is also to be noted that these H10 strains are able to decompose urea, and that 0141 k85 H1 strains are also often urea positive. It is evident from the table that most of the strains are haemolytic.

TABLE 6  
*Biochemical Reactions of the Strains Examined*

|                    | E68<br>0111<br>k85 H11 | G7<br>08<br>K87 H19 | G20b<br>08<br>K87 H19 | G12.3<br>0147<br>K83 H19 | G491<br>0138<br>K81 H19 | CS1522<br>(01 0117)<br>H10 |
|--------------------|------------------------|---------------------|-----------------------|--------------------------|-------------------------|----------------------------|
| Adonitol           | —                      | —                   | —                     | —                        | —                       | +                          |
| Dulcitol           | +                      | +                   | +                     | +                        | +                       | —                          |
| Sorbitol arabinose |                        |                     |                       |                          |                         |                            |
| xylose rhamnose    |                        |                     |                       |                          |                         |                            |
| maltose            | +                      | +                   | +                     | +                        | +                       | +                          |
| Salicin            | + <sup>3</sup>         | —                   | —†                    | —†                       | + <sup>3</sup>          | + <sup>7</sup>             |
| Inositol           | —                      | —                   | —                     | —                        | —                       | —                          |
| Lactose            | +                      | +                   | +                     | +                        | +                       | +                          |
| Sucrose            | —                      | +                   | +                     | +                        | +                       | —                          |
| Mannitol glucose   | ++                     | ++                  | ++                    | ++                       | ++                      | ++                         |
| Sorbose            | —                      | +                   | +                     | +                        | +                       | +                          |
| Urea               | — <sup>4</sup>         | —                   | —                     | —                        | —                       | +                          |
| Haemolysis*        | +                      | +                   | +                     | +                        | —                       | —§                         |

As all strains had the k88(L) antigen this is omitted from the seroformulas given.

\* Some strains of this type are urea positive.

† Both salicin negative and late positive strains of these serotypes have been found.

‡ One of 21 strains received almost simultaneously was haemolytic.

§ Haemolysis tested on 5 per cent sheep blood agar plates.

|| 0138 strains from Germany were haemolytic.

+ means acid ++ means acid and gas.

## DISCUSSION

The first strains with the L antigen K88 were isolated from swine in England. Not only was this L antigen detected in a strain which already had a K antigen of the B type, but the same L antigen was also shown in another strain which differed from the first in respect of O, H and H antigens.

It was later demonstrated that strains having O, B and H antigens (08, k87 H19) similar to one of the two above-mentioned strains, also had the k88 antigen, but of a somewhat different composition. The first k88 antigen to be described was now called k88a, b, whilst the later one was symbolized k88a, c. The fact that apparently identical strains sometimes have the ab and sometimes the ac factors is rather remarkable and leads one to suspect that an antigen variation might take place in the separate cultures, leading to the change from one determinant group of the antigen molecule to the other. This variation phenomenon is well known from the *Salmonella* serology (4).

Variation in the B antigen (or OB complex) in *E coli* has been shown previously, but it has hitherto not been possible to demonstrate this type of variation in the K88 antigen, at least not in the G7 strain. Additional experiments are being carried out.

Another fact worth noticing is that the K88ac factors have been found in several strains of still other serotypes from swine, one of which (0138) is known to be connected with diseases in swine (1). It is known that certain diseases are characterized by certain *E coli* serotypes, e.g. some types are more frequently isolated from the intestinal tract of infants with diarrhoea than from other sources, some are especially isolated from swine with oedema disease (7), some from calves with white scours (8), and the types normally found in the intestinal tract also seem to differ somewhat from animal to animal etc. However, the fact that the L antigen can be associated with a variety of serotypes found in swine is peculiar. In some cases the strains were sent to the International Escherichia Centre because they were primarily shown to agglutinate in a K88 serum, in other words they were selected because of the K88 antigen, whilst in other cases the strains had not been primarily identified serologically at the place of isolation. No investigation has been carried out to determine whether strains with the L antigen K88 can be isolated from sources other than the pig, but it appears from the present study that this L antigen seems to be quite widespread where swine are present, and to be something which can be fixed in different bacterial hosts. This is perhaps not surprising, as it was already known that G7, at least, when mixed with another strain (H509a, not of swine origin), transmits the L antigen to this strain. This ability was not demonstrated in strains with the K88ac factors in the cases examined. Further experiments on these lines will be published later.

The clumping in saline of live cultures of some strains with the L antigen K88 is reminiscent of a similar autoagglutinability in other strains with intact O antigen. Some workers have claimed that K12 and derivatives of this strain clump when they are in the F<sup>+</sup> state (6), from the point of view of an O antigen analysis they are also claimed to be rough, i.e. they lack the O antigen and are autoagglutinable in saline after boiling (9). When the R factor R<sub>100-1</sub> is introduced into a derivative of *E coli* H509a this becomes autoagglutinable, but this property disappears after boiling (2). In these cases the instable live cultures have been shown to harbour an episome (3). The determinant for the K88(L) antigen is probably carried by an episome or a plasmid (5) (unpublished results).

#### SUMMARY

The present study concerns serological examination of strains isolated from swine and shows that

1) The previously described L antigen k88 exists in at least two varieties one of which is symbolized k88<sub>ab</sub> the other k88<sub>ac</sub>

2) After cultivation at 18° C the k88<sub>ac</sub> antigen cannot be demonstrated (the same was previously shown to be true of the k88<sub>ab</sub> antigen)

3) The k88<sub>ac</sub> antigen has been found in several serological types which differed especially as to O and B antigens

4) Strains with the k88<sub>ac</sub>(L) antigen are geographically widely distributed

5) In preliminary experiments it was impossible to show the transferability of the k88<sub>ac</sub> antigen (the k88<sub>ab</sub> factor can be transferred to other strains)

6) The O and H antigens of one of the strains studied were established as new types and numbered 0147 and k89(B)

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## RADIATION RESISTANCE OF *STREPTOCOCCUS FAECIUM* AND SPORES OF *BACILLUS SUBTILIS* DRIED IN VARIOUS MEDIA

By

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Sterilization of disposable items of hospital equipment by means of ionizing radiation has become increasingly employed during the last few years (15, 16)

The calculation of the dose necessary for radiation sterilization of a given group of materials must take into account the greatest possible resistance of the micro-organisms under the given conditions. It is a well-known fact, however, that the resistance of various micro-organisms to ionizing radiation may be altered by variations in the environment before, during, and after the irradiation (8). It must therefore be kept in mind that much of the available information about the resistance of micro-organisms to ionizing radiation is based on the resistance of organisms that have undergone procedures which, *a priori*, cannot be regarded as insignificant for their radiation resistance.

The present study is part of a series of investigations of the resistance of various dried micro-organisms to ionizing radiation. The resistance of two strains of *Streptococcus faecium* and of spores of two strains of *Bacillus subtilis* was investigated before and after the micro-organisms had been subjected to some of the procedures commonly used in bacteriological technique.

### MATERIAL AND METHODS

The strains of *Str. faecium* employed were the two which in a previous study on enterococcal strains (5) were found to be most radiation resistant. One was a laboratory strain ( $\Omega_L$ ) and the other was a sub-strain of a bacterium found in dust from a room housing an electron accelerator used for industrial radiation sterilization ( $\Gamma_1$ ).

The strains were cultured on blood agar at 37° C. for two days. The cultures were then scraped off the plates and suspended in serum broth and isotonic phosphate buffered saline (pH 7.38) respectively. Test pieces for the radiation experiments were made immediately after suspension by drying the suspension as drops (0.01 ml) on polyethylene foil at room temperature in atmospheric air (6).

As previously mentioned two spore forming strains of *B. subtilis* were used. One was the Serum Institute test strain for control of autoclaving and dry heat sterilization. This strain will be designated as *B. subtilis* in the present study. The other

spore forming strain was *B. globigii* ATCC 9372 (*B. subtilis* var. *niger*), which will be designated *B. globigii*. Dried, crude, spore preparations were made as described in a previous study (6). One of the preparations of *B. subtilis* spores, however, was made from a culture grown at 45° C. for five days instead of the usual two days at 37° C. and a further three days at room temperature.

Cleaning of *B. subtilis* spores was performed by suspending the unwashed dry, spore-containing powder in phosphate buffered saline and centrifuging the suspension at 3000 r.p.m. for 30 minutes. This procedure was performed three times, whereupon the sediment was resuspended in phosphate buffered saline, serum broth

a total of ten times. The sediment was then dried at room temperature in atmospheric air and kept in air tight ampoules until use. These washed *globigii* spores were resuspended in distilled water, phosphate buffered saline and serum broth respectively. Test pieces were made from the suspensions immediately after preparation and these test pieces were in every case dry 30 minutes at the most after the

were kept at + 4° C. for 24 hours before use.

In all cases the spore test pieces were made by drying the suspensions in drops (0.02 ml) on polyethylene foil at room temperature in atmospheric air.

The heat resistance of the spores was used as criterion that the spores on the test pieces were actually spores and had not germinated during the preparation procedures. In connection with the determinations of the number of viable units on the

used in the present investigation showed the same or a slightly higher, number of viable units than the test pieces which were not heated.

Before and after irradiation the number of viable units per test piece was determined by suspension in phosphate buffered saline pH 7.38 or chilled serum broth. technique. All counts of viable units

the Research Establishment of the  
the electron accelerator facility or in

plant. The dose in the electron accelerator facility was given with an accuracy of  $\pm 5$  per cent and in the  $Co^{60}$  plant with an accuracy of  $\pm 2$  per cent.

## RESULTS

In the first experimental series, two strains of *Str. faecium* were examined for their resistance to ionizing radiation, after suspension and drying in serum broth, as well as after suspension and drying in phosphate buffered saline. The test pieces compared were prepared from cultures on uniform plates, grown under uniform conditions, and the test pieces were prepared by a uniform technique.

The resistance to ionizing radiation of both strains was less after suspension and drying in buffered saline than after suspension and drying in serum broth, and the reduction in resistance was of the same order of magnitude in both strains (Fig. 1).

Test pieces prepared by suspension and drying of the bacteria in serum broth were stored at room temperature in atmospheric air for



Statens Seruminstitut, Copenhagen and The Danish Atomic Energy Commission  
Research Establishment Risø Denmark

## RADIATION RESISTANCE OF *STREPTOCOCCUS FAECIUM* AND SPORES OF *BACILLUS SUBTILIS* DRIED IN VARIOUS MEDIA

By

LEBBE AHRFENSBURG CHRISTENSEN and KNUD SEHESTED

Received 9 VII 64

Sterilization of disposable items of hospital equipment by means of ionizing radiation has become increasingly employed during the last few years (15, 16)

The calculation of the dose necessary for radiation sterilization of a given group of materials must take into account the greatest possible resistance of the micro-organisms under the given conditions. It is a well-known fact, however, that the resistance of various micro-organisms to ionizing radiation may be altered by variations in the environment before, during, and after the irradiation (8). It must therefore be kept in mind that much of the available information about the resistance of micro-organisms to ionizing radiation is based on the resistance of organisms that have undergone procedures which, *a priori*, cannot be regarded as insignificant for their radiation resistance.

The present study is part of a series of investigations of the resistance of various dried micro-organisms to ionizing radiation. The resistance of two strains of *Streptococcus faecium* and of spores of two strains of *Bacillus subtilis* was investigated before and after the micro-organisms had been subjected to some of the procedures commonly used in bacteriological technique.

### MATERIAL AND METHODS

The strains of *Str. faecium* employed were the two which in a previous study on enterococcal strains (5) were found to be most radiation resistant. One was a laboratory strain (O<sub>12</sub>) and the other was a sub strain of a bacterium found in dust from a room housing an electron accelerator used for industrial radiation sterilization (F<sub>6</sub>).

The strains were cultured on blood agar at 37° C for two days. The cultures were then scraped off the plates and suspended in serum broth and isotonic phosphate buffered saline (pH 7.38) respectively. Test pieces for the radiation experiments were made immediately after suspension by drying the suspension as drops (0.01 ml) on polyethylene foil at room temperature in atmospheric air (6).

As previously mentioned two spore forming strains of *B. subtilis* were used. One was the Serum Institute test strain for control of autoclaving and dry heat sterilization. This strain will be designated as *B. subtilis* in the present study. The other

at  $+4^{\circ}\text{C}$  and a further three days at room temperature

Cleaning of *H. subtilis* spores was performed by suspending the unwashed dry spore-containing powder in phosphate buffered saline and centrifuging the suspension at 3000 r.p.m. for 30 minutes. This procedure was performed three times whereupon the sediment was resuspended in phosphate buffered saline, serum broth and methanol respectively. These were then dried at room temperature.

For the suspension at 8000 r.p.m. for 30 minutes. This procedure was performed a total of ten times. The sediment was then dried at room temperature in atmospheric air and kept in air tight ampoules until use. These washed globigii spores were resuspended in distilled water, phosphate buffered saline and serum broth respectively. Test pieces were made from the suspensions immediately after preparation and these test pieces were in every case dry 30 minutes at the most after the spores had been resuspended. The suspensions were kept at  $+4^{\circ}\text{C}$  for 24 hours and another batch of test pieces was then made from the suspensions. Test pieces of unwashed globigii spores were prepared from suspensions of the spores in distilled water, phosphate buffered saline and serum broth respectively. All these suspensions were kept at  $+4^{\circ}\text{C}$  for 24 hours before use.

In all cases the spore test pieces were made by drying the spores in atmospheric air.

For heat activation all the spore test pieces involved in the present investigation showed the same or a slightly higher number of viable units than the test pieces which were not heated.

Before and after irradiation the number of viable units per test piece was determined by suspension in phosphate buffered saline pH 7.38 or chilled serum broth using the usual dilution and counting technique. All counts of viable units was performed on blood agar.

The irradiations were carried out at Risø the Research Establishment of the Danish Atomic Energy Commission either in the electron accelerator facility or in the  $\text{Co}^{60}$  plant. The dose in the electron accelerator facility was given with an accuracy of  $\pm 5$  per cent and in the  $\text{Co}^{60}$  plant with an accuracy of  $\pm 2$  per cent.

## RESULTS

In the first experimental series, two strains of *Str. faecium* were examined for their resistance to ionizing radiation, after suspension and drying in serum broth as well as after suspension and drying in phosphate buffered saline. The test pieces compared were prepared from cultures on uniform plates grown under uniform conditions and the test pieces were prepared by a uniform technique.

The resistance to ionizing radiation of both strains was less after suspension and drying in buffered saline than after suspension and drying in serum broth and the reduction in resistance was of the same order of magnitude in both strains (Fig. 1).

Test pieces prepared by suspension and drying of the bacteria in serum broth were stored at room temperature in atmospheric air for

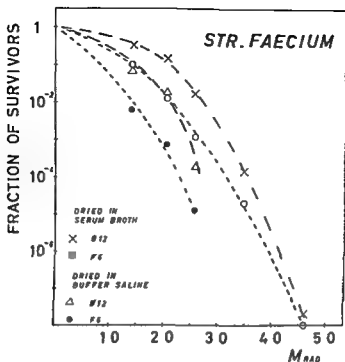


Fig 1

Inactivation curves of two strains of *Str faecium* (O<sub>12</sub> and F<sub>6</sub>) each strain was dried in serum broth and in buffered saline

50 days, and the resistance of the micro-organisms to ionizing radiation was then determined once more. The radiation resistance was unaffected by storage, just as the number of viable units remained unchanged on the non-irradiated test pieces.

Different enterococcal strains can show considerable differences in resistance to storage. In some strains of *Streptococcus faecalis* the number of viable units per non irradiated test piece can fall almost 7 logarithmic steps in the course of 5 months while during the same period the greatest fall observed in the number of viable units on test pieces prepared with *Str faecium* was 3 logarithmic steps. After storage for 11 months a number of the faecium strains studied in a previous investigation (5) showed an unchanged or almost unchanged number of viable units per test piece.

In the second experimental series, washed and unwashed spores of *B. subtilis* were examined for their resistance to ionizing radiation. The spores were obtained from the same preparation, and the washed spores were treated as one lot until suspension and drying in the different media.

Unwashed spores showed the greatest resistance to ionizing radiation after suspension and drying in serum broth (Fig 2). The resistance of the spores was reduced as a result of the washing in phosphate buffered

in methanol had least resistance to ionizing radiation (Fig 3).

Storage of the washed spores as a suspension in serum broth at

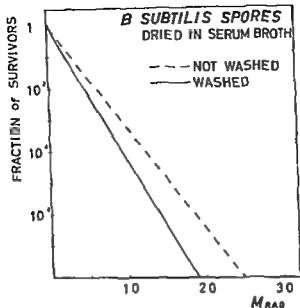


Fig. 2

Inactivation curves of the same spore preparation of *B. subtilis* before and after washing in buffer saline. In both cases the spores were suspended in serum broth and dried in atm. sphere at 1°C of relative humidity.

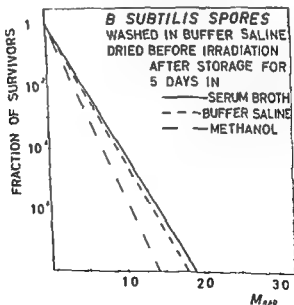


Fig. 3

Inactivation curves  
of spores  
respectively  
for the

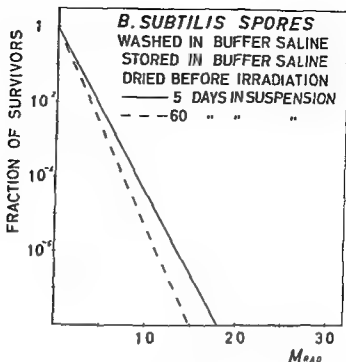


Fig 4

Inactivation curves of washed subtilis spores suspended in buffered saline at  $+4^{\circ}\text{C}$  for 5 days and 60 days respectively

$+4^{\circ}\text{C}$  for 60 days had no effect on the resistance of the spores to ionizing radiation. Storage of the spores in buffered saline, on the other hand, might result in a reduction of the radiation resistance of the spores. After storage for 5 days at  $+4^{\circ}\text{C}$ , spores stored and dried in serum broth showed the same resistance as spores stored and dried in buffered saline, but after storage for 60 days, the resistance of the spores suspended in buffered saline had declined (Fig 4).

Spores suspended and dried in methanol were, as mentioned, the least resistant ones in the experimental series (Fig 3), but their resistance was the same whether they had been suspended in the methanol for 5 days or 60 days. This reduction in resistance was reversible. If the spores were resuspended in serum broth after allowing the methanol to evaporate, they showed the same resistance to ionizing radiation as spores which had been suspended in serum broth for the entire period of storage (Fig 5).

Storage of the spores as suspensions at  $+4^{\circ}\text{C}$  in buffered saline, serum broth, or methanol caused no difference in the number of viable units per ml, whether the number of viable units was determined after heat activation at  $80^{\circ}\text{C}$  for 10 minutes, or whether the determination was made without this heat treatment.

In the third experimental series, was examined whether culture at higher temperature and heat activation at  $80^{\circ}\text{C}$  for 10 minutes had any influence on the resistance to ionizing radiation of subtilis spores.

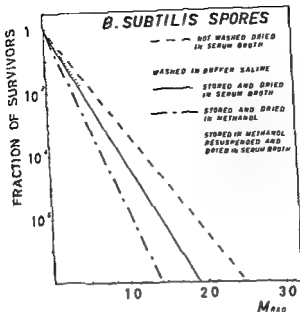


Fig 5

Inactivation curves of the same spore preparation of *B. subtilis*. The washed spores had been stored as suspensions at +4° C for 60 days

Two spore preparations were made concurrently, the culture temperature being the sole difference in the experimental conditions. One culture was grown at 45° C for 5 days, the other at 37° C for 2 days and at room temperature for 3 days. Both radiation resistance and number of spores per mg of dry matter were the same in the two preparations. The only difference which could be demonstrated was that in the preparation grown at 45° C, non-irradiated cultures germinated more rapidly and more uniformly on blood agar than in the preparation grown at the lower temperature. This difference disappeared if the latter preparation was heat activated at 80° C for 10 minutes.

In both preparations, heat activation before preparation of the test pieces was without influence on the resistance of the spores to irradiation. Heat activation after irradiation was of no significance to the number of spores capable of multiplying, and seemed otherwise to be of no value, as both delay in colony formation and inequality of the colonies remained uninfluenced by heat activation at 80° C for 10 minutes in the experiments described here. These two phenomena are always seen after irradiation with high doses, being the more pronounced the higher the dose. Heat activation as well as plating were performed 24 hours at the earliest after irradiation, and thus, the heat-sensitivity of the spores was not examined immediately following irradiation.

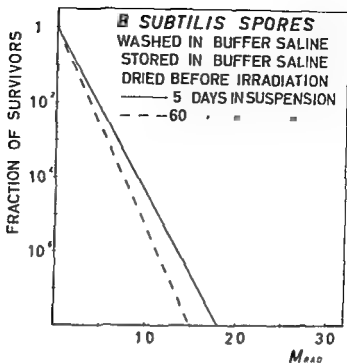


Fig. 4

Inactivation curves of washed subtilis spores suspended in buffered saline at +4° C for 5 days and 60 days respectively

+4° C for 60 days had no effect on the resistance of the spores to ionizing radiation. Storage of the spores in buffered saline, on the other hand, might result in a reduction of the radiation resistance of the spores. After storage for 5 days at +4° C, spores stored and dried in serum broth showed the same resistance as spores stored and dried in buffered saline, but after storage for 60 days, the resistance of the spores suspended in buffered saline had declined (Fig. 4).

Spores suspended and dried in methanol were, as mentioned, the least resistant ones in the experimental series (Fig. 3), but their resistance was the same whether they had been suspended in the methanol for 5 days or 60 days. This reduction in resistance was reversible. If the spores were resuspended in serum broth after allowing the methanol to evaporate, they showed the same resistance to ionizing radiation as spores which had been suspended in serum broth for the entire period of storage (Fig. 5).

Storage of the spores as suspensions at +4° C in buffered saline, serum broth, or methanol, caused no difference in the number of viable units per ml, whether the number of viable units was determined after heat activation at 80° C for 10 minutes or whether the determination was made without this heat treatment.

In the third experimental series, was examined whether culture at higher temperature and heat activation at 80° C for 10 minutes had any influence on the resistance to ionizing radiation of subtilis spores.

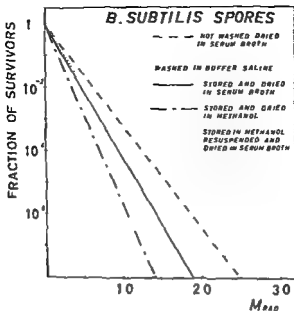


Fig 5

Inactivation curves of the same spore preparation of *B. subtilis*. The washed spores had been stored as suspensions at +4° C for 60 days.

Two spore preparations were made concurrently, the culture temperature being the sole difference in the experimental conditions. One culture was grown at 45° C for 5 days, the other at 37° C for 2 days and at room temperature for 3 days. Both radiation resistance and number of spores per mg of dry matter were the same in the two preparations. The only difference which could be demonstrated was that in the preparation grown at 45° C, non-irradiated cultures germinated more rapidly and more uniformly on blood agar than in the preparation grown at the lower temperature. This difference disappeared if the latter preparation was heat activated at 80° C for 10 minutes.

In both preparations, heat activation before preparation of the test pieces was without influence on the resistance of the spores to irradiation. Heat activation after irradiation was of no significance to the number of spores capable of multiplying, and seemed otherwise to be of no value, as both delay in colony formation and inequality of the colonies remained uninfluenced by heat activation at 80° C for 10 minutes in the experiments described here. These two phenomena are always seen after irradiation with high doses, being the more pronounced the higher the dose. Heat activation as well as plating were performed 24 hours at the earliest after irradiation, and thus, the heat-sensitivity of the spores was not examined immediately following irradiation.



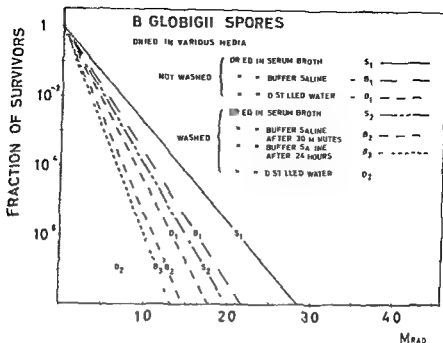


Fig 6

Inactivation curves of the same spore preparation of *B globigii* suspended and dried in various media

In the fourth experimental series, washed and unwashed spores of *B globigii* were examined for their resistance to ionizing radiation, after suspension and drying in various media. The spores were from the same preparation, and were treated concurrently throughout the whole experimental series. As mentioned, the cleaning of the globigii spores was carried out much more effectively than the cleaning of the subtilis spores used in the second experimental series.

Just as in the second experimental series, cleaning of spores resulted in a reduction in their resistance to ionizing radiation (Fig 6). The only outstanding difference between results from the second and the fourth experimental series was that globigii spores suspended in buffered saline and dried within the first 30 minutes of suspension, showed a lower resistance to ionizing radiation than globigii spores suspended and dried in serum broth while, as mentioned, the less effectively washed subtilis spores had the same resistance after 5 days in the same two media.

The washed globigii spores were therefore examined with a view to determining whether they had the same resistance after storage for 24 hours at +4° C, and immediately after suspension. This was found to be the case of globigii spores suspended and dried in serum broth, as well as of spores suspended and dried in distilled water, while the resistance of spores suspended and dried in buffered saline had decreased slightly (Fig 6).

## DISCUSSION

The present study makes no attempt to contribute to an elucidation of the reasons why the radiation resistance of micro-organisms changes as a result of variations in outer conditions. Some of these reasons are familiar. Oxygen tension during and after irradiation is significant for the number of micro-organisms capable of multiplying (11, 20, 22). The water content of the organisms can also be of significance (17, 19, 20, 21), as well as the presence of various substances possessing the capacity of protecting the organisms (3).

The present studies were carried out, because in the literature on radiation sterilization (4, 10, 16), the values quoted for the resistance of various micro-organisms were low in comparison with the radiation resistance of the same organisms in the dry state, when determined by the technique used in the present study (6).

The probability of a number of micro-organisms surviving on material subjected to a sterilization procedure, is in the first instance determined by 1) the sort and number of micro-organisms on the material prior to the sterilization procedure, and by 2) the effect of the inactivating procedure at a point on the material at which the effect is at a minimum.

When sterilization is carried out by autoclaving or by dry heat, the recommendation is to use temperature-time relations which ensure very great inactivation factors for the most heat resistant ones of the generally occurring, contaminating bacteria, even when these organisms are placed at points in the material where the effect of the sterilization procedure is at a minimum (13, 14). If the effect of radiation sterilization is to be equated with the effect of heat sterilization, the dose used for radiation sterilization must be given as a minimum value, as the difference between least dose and mean dose can be of considerable magnitude. Furthermore, the effect of the irradiation must be calculated on the basis of the maximum resistance of the micro-organisms under the given conditions.

As mentioned, the species of the micro-organisms which prior to sterilization may occur on the material is of considerable significance to the inactivation effect which can be achieved by a given sterilization procedure. In radiation sterilization of dry plastic material, it may be permissible to disregard the possibility of an occurrence on the material of *Micrococcus radiodurans* (1), which has a very high radiation resistance in the dry state (6), as this micro-organism so far has been demonstrated only in a few instances. It has been found, however, that *M. radiodurans* is not the only micrococcus which is more resistant than bacterial spores at doses applicable in radiation sterilization of plastics (7). As demonstrated in the present study, some strains of bacteria belonging to *Str. faecium* have such a high radiation resistance if dried in serum broth, that the inactivation factor at 2.5 Mrad is only between  $10^2$  and  $10^3$ . *Str. faecium* is very widespread, and has a considerable

power of survival in the dry state. If it is desirable to maintain the definition of "sterile" as *free from living micro-organisms*, it is necessary for the establishment of doses for radiation sterilization, to take into account the highly radiation-resistant vegetative bacteria.

In England (16) and USA (2), 2.5 Mrad is used, both for sterilization of dry plastic material for use in hospitals, and for sterilization of suture material. In defence of sterilization with 2.5 Mrad, it may be claimed that dry plastic material, as a result of the manufacturing procedure, may be considered contaminated only to a very slight degree before sterilization, always provided that the material is collected and packed under controlled, aseptic conditions, as contamination by dust or touch may involve the transfer of a considerable number of organisms (12).

If radiation sterilization is to give the same inactivation factor for *Str. faecium* on dry plastic material as is usually recommended for test strains such as *B. subtilis* or *B. stearothermophilus* by autoclaving or for *B. globigii* by ethylene oxide sterilization (12), then, based on present knowledge, *the minimum dose in radiation sterilization should be 4.5 Mrad*. This dose is now being used for sterilization of disposable items of hospital equipment at Risø, the Research Establishment of the Danish Atomic Energy Commission.

Were a presence of micro-organisms with a higher resistance to ionizing radiation to be observed at some future date on material to be sterilized by radiation, a standard of radiation sterilization which is in line with that for material autoclaved *lege artis*, could only be achieved 1) if the dose can be further increased, or 2) if the initial contamination can be controlled and kept at a low level, or 3) if the irradiation is combined with other methods of sterilization or disinfection.

When sterilization is carried out by means of continuous irradiation in a high power electron accelerator facility, the dose cannot be made much higher than 4.5 Mrad, as some of the types of plastic usually employed are intolerant to higher doses, when the dose is given in one operation. In most cases, however, the damage to the material seems to be the result of the heat developed during irradiation with these high dose-rates. Intermittent irradiation allows the temperature rise to be controlled, and such change in the irradiation technique may therefore provide the opportunity of raising the dose above 4.5 Mrad without the necessity of using types of plastic which possess greater resistance.

#### SUMMARY

The radiation resistance has been determined on two strains of *Streptococcus faecium* and on spores of two strains of *Bacillus subtilis* dried in atmospheric air after suspension in various media and storage for various periods. In addition, the radiation resistance of the spores was determined before and after cleaning and after culture at 45° C. Resistance to ionizing radiation of *Str. faecium* as well as *subtilis*

spores were found to be highest if they were dried in a protein-containing milieu. Unwashed spores had a greater resistance than washed spores. Culture at higher temperature and heat activation at 80° C for 10 minutes had no influence on the resistance of the subtilis spores.

In relation to the present study, it is recommended that doses in radiation sterilization should be indicated as minimum doses and, if equality is to be established between the effect of radiation sterilization and the effect of heat sterilization, it should be based on the maximum resistance of the most resistant ones of the commonly occurring micro-organisms, under the conditions prevailing during the given sterilization procedure.

A dose of 2.5 Mrad is used at several centres as a routine dose for sterilization of medical equipment. This dose gave an inactivation factor of only  $10^2$  to  $10^3$  for the faecium strains studied, when the strains were dried in serum broth. This factor is very low in comparison with the effect of autoclaving under the conditions usually recommended. At Risø the Research Establishment of the Danish Atomic Energy Commission, the sterilizing dose for disposable items of hospital equipment is at present 4.5 Mrad.

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## BRIEF REPORT

### REGENERATION OF PARIETAL PERITONEUM

By Gunnar Eskeland

Reconstitution of mesothelial defects has been considered to take place

- a) from intact mesothelium surrounding the wound (3),
- b) from mesothelial cells detached from peritoneum and implanted on the wound as free grafts (2)
- c) by metaplasia of cells in the connective tissue underlying the wound (1) and
- d) by a combination of these mechanisms (4)

This report deals with an investigation in which the following experiments were performed

1 Wounds were made in the parietal peritoneum of rats either by burning at 59°C for 15 seconds or by the excision of circular areas including peritoneum the underlying connective tissue and part of the muscle. The wounds were of three different sizes with diameters 4 mm 12 mm and 36 mm. After periods varying from 1 hour to 14 days the abdominal wall carrying the wounds was excised. The peritoneal surface was treated with a weak silver nitrate solution after which the alcohol Hauthen pre  
Efskind (3) and were  
ixed in buffered O<sub>2</sub>

Sections for light and electron  
microscopy were cut on a Huxley microtome

2 Rats in which excision wounds had been made were injected with colcermid (Ciba Basle) 1 mg/200 g body weight 4 hours prior to sacrifice. The abdominal wall carrying the wounds was excised 1-8 days after the wounds had been made and Hauthen preparations were produced as in the previous experiment

3 Small plastic discs made of Vestopal W were placed in the serosal cavities of rats in which the  
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#### Results

1 As early as 1 and 4 hours after the wounds had been made large numbers of cells were observed resting on a fibrin scaffolding and more or less completely covering the surface of the wound. These cells were of the same appearance as the free peritoneal fluid cells of the monocyte/macrophage type and were present before

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# BRIEF REPORT

## PHOTOINACTIVATION OF VISNA VIRUS

B) Halldor Thormark and Inger Petersen

Viruses show a marked variation in their sensitivity to visible light applied in the presence of photodynamic dyes and it has been suggested that the rate of photodynamic inactivation of a virus is a characteristic property which might be useful for taxonomic classification of newly isolated animal viruses (1, 2).

In an attempt to characterize visna virus a recently isolated virus of sheep (3) a number of its physical, chemical and biological properties has been studied including its sensitivity to visible light in the presence of toluidine blue. The virus was grown in cultures of sheep cells in medium 199 with 1 per cent sheep serum (4) and was diluted tenfold in phosphate buffered saline (without Ca and Mg) pH 7.3. The irradiation procedure was similar to that employed by Hall *et al* (1). Toluidine blue (Merck) was added to the virus in a final concentration of 6 µg per ml ( $2 \times 10^{-5}$  M) and the mixture was kept in the dark at 4° C for 45 minutes. Twenty five ml of the mixture were then poured into an aluminium dish 65 mm in diameter and irradiated with a 250 W Osram Nilraphot SR lamp at a distance of 70 cm from the bottom of the dish. During irradiation care was taken to secure an adequate stirring and temperature control of the fluid. Samples of 0.3 ml were taken at intervals, diluted tenfold in Hanks solution and dialysed in the dark against two changes of Hanks solution before titration of surviving virus.

Vaccinia virus (control)

1. Toluidine

in culture

was added

before

the end

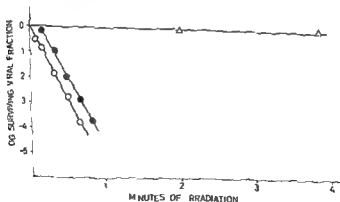


Fig 1

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an appreciable cellular infiltration could be demonstrated in the tissues underlying the wound. None of the cells observed within the limits of the defect at these early stages resembled mesothelial cells.

From 2 days onward single cells or islands of cells with the characteristics of mesothelium were found scattered over the wound surface. These cell islands continuously increased in size until even the largest wounds were completely covered with mesothelium at 8-10 days.

2. In rats treated with colcemid numerous mitoses were found both in the mesothelial like cells scattered over the wound surface and in a narrow brim of intact mesothelium bordering on the wound. In both locations mitoses were numerous at 3 days and maximum mitotic activity was recorded 3 and 4 days after the wounds were made.

3. The plastic discs were partly or completely covered with cells in one or several layers. The superficial layer was composed of flattened cells which in many instances formed continuous sheets with the characteristics of mesothelium.

4. The central filters of the diffusion chambers were partly or completely covered by cells which were of two main types. Typical fibroblasts and cells with the same appearance as proliferating mesothelium seen in healing peritoneal wounds.

### Conclusions

Free mononuclear cells from the peritoneal fluid may become implanted on to the surface of peritoneal wounds and have the potentialities of differentiating to form mesothelial cells. These cells multiply and are considered to be a major source of the mesothelial cells ultimately covering the defect.

The intact mesothelium in the immediate vicinity of the wound also participate in the repair process by proliferation and ingrowth.

Detailed reports of the experiments will be published.

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## TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting September 26, 1964

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### SYMPOSIUM ON MODERN METHODS IN ROUTINE HISTO-PATHOLOGY

S Falkmer HISTOCHEMISTRY

J Ahlqvist ENZYME HISTOCHEMISTRY

The mechanisms of the metal the azo dye the indigogenic and the tetrazolium techniques for demonstrating enzymes in tissue sections were described. Some of the difficulties in the interpretation of results were discussed amongst others those caused by uneven distribution of substrates in the tissues. This makes quantitation of reactions difficult as far as regards the number of reactive sites. It does not, however constitute a drawback for enzyme histochemistry since the forces resulting in the uneven distribution may be the same as those leading to the formation of the enzyme substrate complex at least in some cases. The uneven distribution of substrates thus might include part of the mechanism of enzyme activity. Certain hitherto obscure findings in enzyme chemistry might be explained if the local concentration of substrate accessible to the action of reactive sites sometimes were dependent on structures other than the molecule carrying the reactive site. With regard to the function of enzymes *in vivo* this would imply that it sometimes might be preferable to carry out enzyme studies on as intact tissues as possible.

L Esposti CYTOCHEMICAL STUDIES ON PROSTATIC CARCINOMA

M Moberger SULPHYDRYL GROUPS AND CANCER

A Ljungquist JUXTAGLOMERULAR GRANULES

B Engfeldt X RAY METHODS

A Bergstrand ELECTRON MICROSCOPY

F Linell THE DIAGNOSIS OF PARASITIC DISEASES

B Fors STAINING OF FUNGI

G Nathorst Wing Jahl STAINING OF PNEUMOCYSTIS

During the last few years endemics and isolated cases of Pneumocystis pneumonia have occurred in Sweden and 20 deaths have been recorded.

of visna virus was almost identical to that of vaccinia virus 90 per cent of the infectivity being lost in about 10 seconds. Poliovirus was not significantly inactivated by irradiation for 4 minutes. Preincubation with toluidine blue in the dark at 4° C for 2½ hours instead of 45 minutes before irradiation did not cause a more rapid inactivation of visna virus. Incubation with dye in the dark throughout the experimental period did not cause a significant inactivation of either visna or vaccinia virus.

The present experiment shows that visna virus is relatively sensitive to photodynamic inactivation under the conditions employed and equally sensitive as vaccinia virus. Poliovirus on the other hand was stable and has recently been found to require other conditions in order to be inactivated (8). The reason for the differential sensitivity of viruses to photodynamic action is not known. Hiatt (2) has suggested that it reflects differences in permeability of the viral protein coat to penetration of dye. The visna virus particles are rather incompact being coated by a thin membrane apparently derived from the cytoplasmic membrane of the host cell (5). Vital dyes like toluidine blue may therefore have an easy access to nucleic acid in the interior of the virus explaining that maximal rate of photodynamic inactivation is observed after preincubation of virus and dye in the dark for 45 minutes or less at 4° C, pH 7.3. The size and external structure of visna virus is apparently similar to that of influenza virus and avian and mouse tumour viruses (5, 6). Photoinactivation of influenza virus has been reported recently under conditions somewhat different from those employed in the present study (7, 9).

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For the identification of the implicated organism *Pneumocystis carinii* the following stains are recommended PAS gallocyanin chromalum staining and either a STB method (sulphation toluidine blue method) or a modification of Gomori's methenamine silver nitrate procedure. The STB stain used is a modification of the procedure described by *Chalardjian & Graue*. The sections are deparaffinized in xylene which is washed off in ether, and then placed in a sulphuric acid ether reagent at  $+4^{\circ}\text{C}$  for 15 minutes. After washing in water the slides are placed in 0.06 per cent toluidine blue (Hoyer Merck) for 15 minutes, washed in water, dehydrated in isopropylalcohol, cleared in xylene and mounted.

With the PAS gallocyanin chromalum staining the membranes of all cyst forms stain red and the internal structures grey blue. The STB procedure stains thick walled cysts metachromatically violet with the methenamine silver nitrate technique the thick walled parasites stain grey black.

For smears the Giemsa method and the STB procedure are recommended. With the Giemsa stain the internal structures of the parasites are visualized whereas the STB method stains the membranes.

*G. Moberger* FINE NEEDLE ASPIRATION BIOPSY

*S. Franzen* FINE NEEDLE ASPIRATION BIOPSIES IN THE NECK REGION

*S. Dahlgren* INTRA-THORACIC NEEDLE ASPIRATION BIOPSIES

*J. Ponten* TISSUE CULTURE

*J. Mellgren* TISSUE CULTURE ON CARCINOMA IN SITU AND INVASIVE CARCINOMAS OF THE CERVIX

*B. Stenkvist* BENIGN AND MALIGNANT FIBROBLASTS IN VITRO

Oslo University Institute of Pathological Anatomy Rikshospitalet Oslo Norway  
 Prof O Torgersen MD) Histochemical Laboratory (S D Schultz Haudt PhD)

# COMPARATIVE HISTOCHEMISTRY OF NON INJURED SKIN CONNECTIVE TISSUE OF NORMAL AND ASCORBIC ACID DEFICIENT GUINEA PIGS

By

STIG D SCHULTZ HALDT and SIGURD HJ FROM

Received 29 11 64

The relation of ascorbic acid to connective tissue metabolism has been the subject of much study particularly with respect to the function of vitamin C in the biosynthesis and maintenance of collagen (Gould 1963) There are however, also many reports describing alterations in ground substance polysaccharides in ascorbic acid deficiency Mostly these have been concerned with changes occurring in healing wounds or carrageenin induced granulomas in normal as compared to vitamin C deficient guinea pigs and the observations made have been conflicting (Gould 1963)

The investigations here presented represent efforts to establish quantitative differences in the polysaccharide contents of non injured skin connective tissues of normal and vitamin C deficient animals by a combination of histo and biochemical techniques

## MATERIALS AND METHODS

Young guinea pigs weighing 250 g were used in the experiments Some of the animals were maintained on an ascorbic acid deficient diet until they had suffered considerable losses in body weight usually for 18 to 20 days These and control animals were then treated in an identical manner They were anaesthetized by ether inhalation and the hairs of the trunk were clipped whereafter the animals were sacrificed

As possible  
 sized

Portions of the dried extracts were then subjected to paper electrophoresis on Whatman no 3 MM material were dried but were subjected to electrophoresis temperature

This investigation was supported by Grant no DI 01472 03 from the NIDR of the National Institutes of Health Bethesda Maryland U S A

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Portions of the dried extracts were then subjected to paper electrophoresis on Whatman no 3 MM filter paper strips measuring 57 by 4 cm (small) and -  
 material were dissolved in 110 µl -  
 solutions were applied across the -  
 Electrophoresis was carried out w -  
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Fig 1

The figure represents a photograph of stained paper strips after electrophoresis of skin extracts from normal and vitamin C deficient guinea pigs. The two upper strips are stained with toluidine blue, the two lower by means of PAS. The first and third strips from the top represent the results obtained with normal skin extract, the second and fourth those with the extract from ascorbic acid deficient animals. The component with the greatest mobility towards the anode (+) contained chondroitin sulphates, the slower moving one hyaluronate, whereas the slowest migrating component represents glycoprotein. In all cases identical amounts of the extracts had been applied to the paper strips prior to electrophoresis.

was found not to be confined to any one particular type. Following incubation with the hyaluronidase, a zone representing chondroitin sulphate B showed up after electrophoresis and staining, although the colour intensity of the zone was fainter than with identical amounts of undigested extracts.

With regard to glycoproteins as demonstrable by means of PAS, it appeared that there were considerably smaller amounts of such materials present in normal skin extracts (the second strip from the bottom in Fig 1) than in identical amounts of extract from vitamin C deficient animals (the bottom strip).

The impressions gained from the paper electrophoresis experiments were confirmed by the chemical analyses. The results of these are shown in Fig 2.

As seen from Fig 2, there was slightly more total acid mucopolysaccharide material present in the extract of normal skin than in that of the skins of the ascorbic acid deficient animals. Under normal conditions, there were 1.7 times as much chondroitin sulphate as hyaluronate (in terms of galactosamine and glucosamine, respectively). In ascorbic acid deficiency, on the other hand, there was twice as much hyaluronate present as chondroitin sulphate. With regard to the heteropolysaccharides of the glycoproteins, the extracts from the vitamin C deficient animals contained nearly four times as much as did those from normal animals.

Thus the results showed that in ascorbic acid deficiency, there is more ground substance polysaccharide material present in the skin connective tissue than under normal conditions. This increase is due to an accumulation of "neutral heteropolysaccharide" containing materials, *i.e.* glycoproteins. In vitamin C deficiency, there is also more hyaluronic acid present than under normal conditions. The total

Identical amounts of dried extracts were exposed to testicular hyaluronidase (Wydase Wyeth Inc) M acetate buffer of pH reducing units of the e running tap water, lyophilized and subjected to paper electrophoresis as above.

Following electrophoresis the paper strips were dried at 100° C and one of each series divided lengthwise into two 2 cm wide ribbons. One of these was stained with toluidine blue in order to locate hyaluronic acid and chondroitin sulphuric acids (Schultz Haudt *et al* 1964), whereas the other was stained by the periodic acid Schiff technique (PAS) as described by Ironsson (1961) for the localization of glycoproteins. Areas corresponding to the stained zones on the ribbons were cut out from the remaining, unstained strip and eluted with water. The eluates were dialysed and subsequently lyophilized.

The amounts of glycoprotein present in the eluates were determined by the colourimetric PAS method of Hooghwinkel & Smits (1957).

Dried eluates from the zones corresponding to those that stained with toluidine blue were hydrolysed with 1 ml of 6 N HCl for 4 hours at 110° C in sealed test tubes. The hydrolysates were treated with charcoal filtered and evaporated to dryness by distillation at reduced pressure and a temperature of 40° C. They were next redissolved in water, and the solution added onto a column (0.9 by 10 cm) of a cationic exchange resin (Dowex 50 H<sup>+</sup>, X-12). Hexosamines were eluted from the column with 0.5 N HCl. 10 ml being collected. The eluates were lyophilized. They were finally redissolved in appropriate amounts of water and subjected to paper chromatography (Stoffyn & Jeannel 1954) and a colourimetric reaction (Roseman & Daffner 1956) in order to determine the presence and amounts of glucosamine (representative of hyaluronate) and of galactosamine (representative of chondroitin sulphates).

The incubation with hyaluronidase was carried out for control purposes as well as in order to differentiate between chondroitin sulphate B and the A and/or C varieties of this acid mucopolysaccharide. The enzyme preparation used catalysed the hydrolysis of hyaluronate as well as that of chondroitin sulphates A and C but not that of type B.

## RESULTS

Typical, stained electrograms are shown in Fig 1. As seen in the figure, the extracts from both normal and ascorbic acid deficient animals divided upon electrophoresis into 3 polysaccharide containing components, all migrating towards the anode. The slowest migrating one stained by PAS only (the two lower strips) and, hence, contained heteropolysaccharide material characteristic of glycoproteins, but different from the acid mucopolysaccharides (Schultz-Haudt *et al* 1964). The two faster migrating components reacted with toluidine blue only (the two upper strips in Fig 1). The slowest of the two contained glucosamine and disappeared after incubation of the extract with hyaluronidase. It, therefore, was judged to contain hyaluronic acid. The fastest moving one contained galactosamine, but was only partially susceptible to the action of the enzyme. Hence, this component was considered to contain a mixture of chondroitin sulphates C (and/or A) and of B (resistant to the enzyme preparation). By visual comparisons of the strips—onto which identical amounts of the extracts had been applied—it appeared that normal skin (the strip on top of Fig 1) contained considerably more of chondroitin sulphates than of hyaluronate. This situation appeared reversed in vitamin C deficiency (the second strip from the top). In similar experiments, the reduction in the contents of chondroitin sulphates in the ascorbic acid deficient animals

Firstly, since most of the earlier investigations were based on studies of healing wounds of the carrageenin induced granuloma, or of tissue growth in polyvinyl sponge implants. Secondly, because many previous studies have been strictly histochemical in nature, and evaluations of colour intensities in tissue sections as observed in the light microscope are uncertain except in extreme cases. Thirdly, because there has in the past been uncertainties about the chemical basis for some of the methods used in connective tissue polysaccharide histochemistry.

With regard to the latter point the present as well as other investigations (Schultz Haudt *et al* 1964) have demonstrated that so far as skin and mucous membranes are concerned a differentiation between glycoproteins (containing the so-called "neutral heteropolysaccharides") and mucoproteins (containing acid mucopolysaccharides) may be made by means of the PAS technique and the toluidine blue stain, respectively.

In relation to the second point the method used in the present experiments permits for fairly satisfactory quantitative comparisons. Moreover, a distinction may be made between types of chondroitin sulphates.

Taking the above considerations into account, the present findings are in agreement with the previous suggestions made (Gersh & Catchpole 1949 1960, Persson 1953) to the extent that there is an increase in ground substance in scurvy. This increase, however, is due to an accumulation of glycoprotein material. With regard to total mucopolysaccharide—in terms of hyaluronate and chondroitin sulphates—a slight decrease was found in vitamin C deficiency. This is in conflict with some earlier observations (Bunting & White 1950 Bradfield & Kodicek 1951 Persson 1953 Dunphy *et al* 1956 Robertson & Hinds 1956 Kimoto *et al* 1959). The apparent disagreement is however, at least in part, resolvable by considering the two types of acid mucopolysaccharides under consideration separately. As shown by the present results, there is an increase in hyaluronic acid in ascorbic acid deficiency, as compared with the normal situation. This is in agreement with observations made on healing scorbutic wounds (Gould 1963) and on the carrageenin induced granuloma (Slack 1957 1958). On the other hand, there is a marked decrease in the amounts of chondroitin sulphates in vitamin C deficiency accounting for the slight reduction in total acid mucopolysaccharide. That reduced amounts of chondroitin sulphates are present in scurvy as compared to normal conditions is in accordance with the conclusions arrived at by Kodicek & Ioeft (1955) with respect to healing tendons by Slack (1958) in his studies of the carrageenin granuloma, by Stein & Wolman (1958) in their investigations of healing wounds, and by Hughes & Kodicek (1960) experimenting with granulation tissue. It has been suggested that the ground substance polysaccharides in scurvy are depolymerized and that they are more soluble than under normal conditions (Gersh & Catchpole 1949 Persson 1953). In the pre-

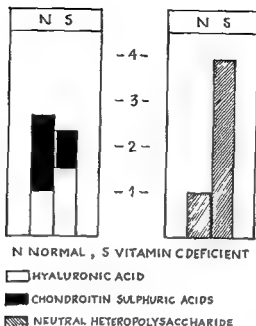


Fig. 2

A schematic illustration of the results obtained with respect to the amounts of the examined polysaccharides present in extracts from normal and vitamin C deficient guinea pig skin. The unit is selected at random and does not refer to amounts present on for example a dry weight basis. Hence in terms of the total amounts of the types of polysaccharides present the two halves of the figure are not comparable. Total acid mucopolysaccharide was arrived at by adding the measured amounts in eluates from the paper electrophoresis strips of glucosamine (= hyaluronic acid) and galactosamine (= chondroitin sulphuric acids). In spite of the increase in hyaluronic acid in ascorbic acid deficiency there was a slight decrease in total acid mucopolysaccharide due to the reduction in the amounts of chondroitin sulphuric acids. The colourimetric measurements of neutral heteropolysaccharides showed a great increase of such material in vitamin C deficiency. An absolute quantitation was impossible due to the lack of suitable reference substances.

amounts of acid mucopolysaccharides present in ascorbic acid deficiency, however, are somewhat reduced as compared with normal animals due to a considerable reduction in chondroitin sulphates.

For control purposes, the collagen contents of skin homogenates as well as of the extracts from the two groups of animals were determined in terms of the contents of hydroxyproline following hydrolysis (I each 1960). The much higher amounts of this amino acid present in the materials from the normal animals were considered indicative of true ascorbic acid deficiency in the experimental group of guinea pigs.

## DISCUSSION

The results here presented show that ascorbic acid deficiency causes definite alterations in the polysaccharide composition of the ground substance of otherwise non-injured guinea pig skin connective tissue. The results, however, appear not entirely comparable to most of the observations made previously by other investigators, for several reasons

Firstly, since most of the earlier investigations were based on studies of healing wounds, of the carrageenin induced granuloma, or of tissue growth in polyvinyl sponge implants. Secondly, because many previous studies have been strictly histochemical in nature, and evaluations of colour intensities in tissue sections as observed in the light microscope are uncertain except in extreme cases. Thirdly, because there has in the past been uncertainties about the chemical basis for some of the methods used in connective tissue polysaccharide histochemistry.

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It has been suggested that the ground substance polysaccharides in scurvy are depolymerized and that they are more soluble than under normal conditions (Gersh & Catchpole 1949, Persson 1953). In the pre-

sent experiments, no efforts were made to evaluate these suggestions. However, the respective rates of migration of the various polysaccharide components upon electrophoresis were identical whether they derived from ascorbic acid deficient animals or not. The possibility of increased solubilities in scurvy may be thought to be reflected in the obtained data with regard to hyaluronic acid and glycoproteins. As a control, tissue residues after NaCl extraction were further extracted with 0.5 N NaOH, and the extracts were analysed for the presence of hexose and hexosamine. The analyses showed that the NaCl extraction had not been entirely exhaustive in terms of removing all sugar containing components. On the other hand, the amounts of such materials left over were not sufficiently large to significantly influence the results presented.

An interpretation of the observations made, in terms of differences in tissue biochemistry, is difficult. It seems, however, reasonable to suppose that when it comes to the acid mucopolysaccharides, lack of ascorbic acid interferes with the synthesis by the cells of chondroitin sulphates. This interference may be related to a reduced rate of sulphation of precursors (Kodicek & Loevi 1955, Stein & Wolman 1958) although it is a matter of some dispute whether or not the rate of sulphation is affected by ascorbic acid (Friberg 1958, Robertson 1961). More probably, there is a reduction in the rate of synthesis of the chondroitin sulphates in general (Slack 1957, 1959). How this is related to an increased rate of synthesis—or maybe a retarded rate of breakdown—of hyaluronic acid is, at the moment, hard to evaluate.

The excessive accumulation of glycoproteins in vitamin C deficiency may not be primarily related to tissue metabolism. It is suggested that it may, at least in part, be the result of an increased infiltration of the connective tissue by serum glycoproteins because of the injury to the capillaries associated with ascorbic acid deficiency. A suggestion of the kind is in disagreement with Pirani & Latchpole (1951) who observed that the serum glycoprotein concentration increased in scurvy, but felt that this was due to the absorption into the circulation of depolymerized connective tissue polysaccharides.

#### SUMMARY

Ground substance polysaccharides of skin connective tissue of normal and vitamin C deficient guinea pigs have been studied by means of a combination of histo- and biochemical techniques.

As compared to the normal tissue, the skins from the ascorbic acid deficient animals contained

1. considerably more polysaccharide materials. This was found to be due to an accumulation of heteropolysaccharide material characteristic of glycoproteins,

2 slightly less total acid mucopolysaccharide material This was due to a sharp reduction in the amounts of chondroitin sulphates present, 3 more hyaluronic acid, although not enough to compensate for the reduction of chondroitin sulphates in terms of the total amounts of acid mucopolysaccharides present

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## MASSIVE FATAL PNEUMOCYSTIS PNEUMONIA IN LEUKAEMIA

### *Report of Two Cases*

*By*

G NATHORST-WINDAHL, B H HESSELMAN, B SJOSTROM  
and JAN POJTEK

Received 18 II 64

Pneumocystis pneumonia (Pcp) manifests itself as an interstitial pneumonia, often characterized by a plasmacellular reaction. This pulmonary disease has been known in Europe for more than two decades as "interstitial plasma cell pneumonia". In infants it is a serious, often epidemic, disease carrying a high mortality. Pcp is uncommon in older age groups where it ordinarily is of a benign character and usually is detected at autopsy as small circumscribed pneumonias (Mur 1959). Massive fatal Pcp in older children and adults is rare.

Pcp and the aetiological rôle and biological nature of the implicated organism *Pneumocystis Carinii* (PC) have been reviewed (Jirovec & Vaneek, Carlgren & Nathorst-Windahl 1955, Hampert 1956, Gajdusek 1957, Jirovec 1959).

The first case to be described is one of a four year-old girl with acute leukaemia treated with adrenocorticoids and cytostatics for several months. The leukaemia was in remission at death and the immediate cause of death was respiratory insufficiency due to a massive Pcp. The second case is one of a 55-year old man with lymphatic leukaemia of eight years duration. The leukaemia was treated with corticosteroids and cytostatics. In this case the immediate cause of death was also a massive Pcp. Both cases occurred in the same hospital but in different wards.

The cases seem to be of interest not only because of their rarity (they are in fact the first cases of Pcp in older age groups described in Sweden) but also as an illustration of a possible connection between prolonged treatment with corticosteroids and cytostatics and the development of Pcp.

### CASE REPORTS

*Case 1* The patient was a girl born in September 1954 who was admitted to the hospital in August 1958 with a history of a sore throat and fever. Penicillin had been



Fig 1

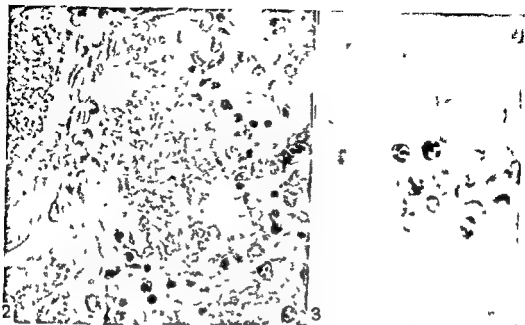
Case 1 *Pneumocystis pneumonia*. The alveoli contain characteristic foamy material. Moderate interstitial broadening and infiltration with chiefly lymphocytes. PAS gallicyanine  $\times 160$ .

Given for a few days. At admission the child was acutely ill with high fever and

at this normal the bone marrow smear examination was rendered difficult by a technically unsatisfactory puncture but the picture strengthened the suspicion of leukaemia. However as a remission seemed to have ensued the patient was discharged two weeks after admission but asked to meet for ambulatory follow-up. The

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Fv  
the marrow smears now showed changes characteristic of acute leukaemia.

As the patient was  
interfered with  
mycin was  
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the girl was discharged with prednisolone and controls  
during the next few months showed a satisfactory course. However the



Figs 2 and 3

Fig 2 Case 1 Pneumocystis pneumonia Honeycombed appearance of alveolar content typical of *Pneumocystis Carinii* infection PAS gallicyanine  $\times 385$

Fig 3 Case 1 Lung Alveolar content with Gram positive *Pneumocystis Carinii* organisms Gram Weigert  $\times 700$

During the following months the course was satisfactory but subsequently a fever and continuous dyspnoea developed. The latter was considered due to a disturbance in the electrolyte equilibrium in connection with the intense protracted corticosteroid treatment. The total quantity of prednisolone and mercaptopurine given was 3870 mg and 5000 mg respectively. The prednisolone was now replaced by dexamethasone (Decadron MSD). However the child's condition became worse and she was readmitted for the last time in May 1979. The patient was now gravely ill and had a rapid laboured and shallow respiration. X-ray examination of the lungs showed widespread small patchy infiltrations and milky shadows on both sides. A disseminated pulmonary candidiasis was suspected since the patient had previously had an oral moniliasis. Mycostatin (Bofors) was administered without effect. Oxygen inhalation had but very slight beneficial effect. Respiratory difficulties and dyspnoea increased and three days after the last admission the patient died.

#### Pathologic Findings

**Lungs.** Grossly the tissue was diffusely consolidated and grey in formal fixed state. The cut surface showed a distinct lobulation. Lighter polygonal areas of a maximal diameter of about 9 mm were surrounded by darker hyaline septa.

Microscopically the diffuse character of the process could be confirmed and no normal areas were found in a large number of sections. The majority of the alveoli were filled by honeycombed faintly eosinophilic masses which were often retracted from the alveolar walls (Figs 1-2). In some areas the alveolar content was more compact and partly had the appearance of hyaline membranes lining the alveoli. A few bronchioles were also filled by honeycombed material. Gram Weigert stainings showed that this material consisted of large numbers of intralveolar Gram positive rounded or crescent like forms of the parasite (Fig 3). Their sizes were 5-7  $\mu$ m. With the silver methenamine method also numerous organisms were seen. PAS gallicyanine stain showed an intense positive reaction in the mucoid envelopes of the parasites.

In such alveoli as were filled by the honeycombed PC colonies the lining cells were cuboid flattened and had partly desquamated. Other alveoli in which the

eosinophilic material was more sparse had prominent lining cells with vesicular nuclei containing nucleoli. Such cells were often desquamated and were lying free in the alveolar lumina. The alveolar septa were often broadened and infiltrated by inflammatory cells. Most of these cells were lymphocytes but granulocytes some of which were eosinophilic and histiocytes were also seen.

With methyl green pyronine stain only small numbers of plasma cells were seen. The inflammatory cells were most numerous in the septa and around blood vessels. The interlobular septa were broadened by oedema. No intranuclear inclusion bodies were seen.

Sudan IV stain showed areas with macrophages and desquamated alveolar cells

These cells were interpreted as blast cells belonging to the myeloid series.

**Adrenal glands** Microscopic investigation showed an atrophic cortex with narrow fasciculate and reticular zones. Sudan IV stain showed a marked reduction of lipid content especially in the zones mentioned.

**Case 2** A man born in July 1905 chronic lymphocytic leukaemia had persisted since 1932. He had been working until 1956 at which time his superficial lymph nodes increased in size. At this time he received treatment with local irradiation. There was a moderate regression of the lymph node enlargement and the white blood cell count decreased from 332000 to 75000 per cu mm.

In March 1959 the patient was admitted to the hospital for an acute otitis media with mastoiditis and purulent meningitis. Mastoidectomy and intense antibiotic

therapy were given. In January 1960 an X-ray examination of the chest showed bilateral pleurisy. There was a moderate fever and X-ray of the chest showed infiltrations in the right lung radiating from the hilar region (Fig. 4). The peripheral lymph nodes were enlarged. The patient received irradiation to the chest and the pleurisy subsided.

In May a rather grave haemolytic anaemia supervened and the patient received prednisone. There was a temporary improvement in the haemoglobin and roentgenograms showed infiltrations in the major part of the lung.

There was no effect of either antibiotics or steroids given in large doses. In November 1959 and July 1960 the relative values for gamma globulin in blood were 65 and 54 per cent respectively which indicated a grave hypogammaglobulinemia. A week before death the patient received 16 ml of gamma globulin without any effect on the respiratory distress.

Terminally the patient had a shallow respiration and cyanosis and he died in respiratory insufficiency in July 1960.

Autopsy revealed heavy and consolidated lungs with a greyish yellow and firm parenchyma. The pleura was of normal appearance. The liver weighed 2500 g and had a normal size.

Histological examination of the lungs showed a picture similar to the first case described. The alveolar septa were thickened by eosinophilic material. The periodic acid Schiff galloxyamine stain coloured the alveolar content very distinctly with PAS positive mucoid envelopes of the parasites and in many of the envelopes small dots and bars of gall exanine positive nuclear material. With Gram Weigert stain isolated cyst or



Fig 4

Case 2 Roentgenogram of chest  
March 1960 Rather dense local-  
ized infiltrations in the right  
lung radiating from the hilar  
region



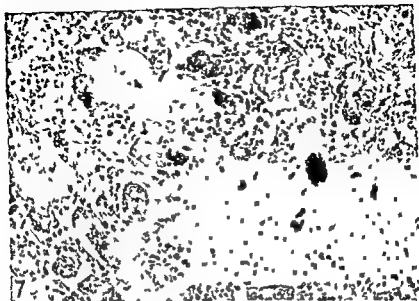
Fig 5

Case 2 Roentgenogram of chest  
April 1960 Rather massive  
streaky infiltrations in both  
lungs radiating from the hilar  
regions



Fig 6

Case 2 Roentgenogram of chest  
July 1960 Streaky and nodular  
confluent infiltrations in the  
major part of the right lung  
Prominent infiltrations also in  
the left lung



Fig

Case 2 *Pneumocystis pneumonia*. All alveoli in the field contain honeycombed colonies of *Pneumocystis Carinii*. Interstitial infiltration chiefly by lymphocytes. PAS gallic aniline  $\times 140$ .

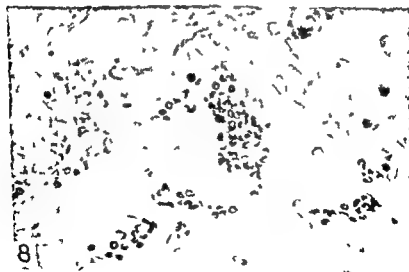


Fig 8

Case 2 Lung. Mass silver impregnated *Pneumocystis Carinii* organisms are seen in rings & crescents. Silver methenamine  $\times 420$ .

crescent formed Gram positive parasites were clearly demonstrated and with the silver methenamine method —

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leukaemic infiltrations with small accumulations of mature lymphocytes in the portal tracts in the liver and in the spleen, a rather diffuse infiltration with lymphocytes

## DISCUSSION

The diagnosis of leukaemia and Pep seems to be established beyond doubt in both cases

In the first case the clinical picture was typical of an acute sub-leukaemic leukaemia and two sternal marrow biopsies contained large numbers of small blast cells interpreted as paramyeloblasts. The leukaemia was evidently in (partial) remission at death and no blast cells were noted in the liver at autopsy and found only in moderate numbers in the spleen

In the second case the diagnosis of leukaemia was based on a positive sternal marrow biopsy and on the white cell count of 332000 per cu mm on one occasion with dominance of mature lymphocytes. There was also enlargement of superficial and mediastinal lymph nodes, the liver and the spleen. At autopsy lymphocytic leukaemic infiltrations were found in the liver and spleen and, to a lesser degree, in the lungs

Pep was not diagnosed *intra vitam* but the histological demonstration of a honeycombed eosinophilic alveolar content with large numbers of PC in the lung section was unequivocal

In spite of the fact that the parasite PC has been known since 1910 and thoroughly investigated with histological and histochemical methods (Carini 1916, Opferkuch 1959) and with the electron microscope (Seifert 1960, Seifert & Pliess 1960) the taxonomic state of the parasite has not been fully elucidated. The organism is usually considered to be a protozoon and has not yet been cultured *in vitro*

Cultures from the lungs were not attempted in the present cases

The inflammatory reaction of Pep in infants is usually characterized by the presence of numerous plasma cells. Our cases differ in this respect since plasma cells were found only in small numbers. This may well be due to the radiation therapy in the second case and to the prolonged steroid treatment in both cases, which factors are known to depress plasmacytogenesis and production of antibody. Case 2 also showed a pronounced hypogammaglobulinaemia thus providing interesting analogies to the reports of Pep in infants with agammaglobulinaemia (Kramer 1962)

In retrospect the respiratory distress during the last days of life in both of our cases taken together with the roentgenological lung picture

and the insignificant physical findings in the lungs are typical clinical signs of Pep (Eberling & Cohen 1958, Schafer 1955)

Whether the combination of leukaemia and Pep was only fortuitous or whether a pathogenetic connection existed is of great interest. Recent observations indicate that serious diseases affecting the reticuloendothelial system may lessen the resistance against PC. Circumscribed Pep has thus been diagnosed at autopsy in adults with Hodgkin's disease (Mur 1959, Vanek 1953) and various forms of leukaemia (Hendry & Patrick 1962, Jirovec & Vanek 1954)

In addition to the possible rôle played by leukaemia *per se* in preparing the ground for Pep, the intense treatment with corticosteroids, antibiotics and cytostatics was conceivably of importance (Kossel 1962, Muller 1960, Roos & Keller 1960)

It is well known that corticosteroids, especially when combined with antibiotics, lessen the resistance against infection, and evidence of a connection between administration of adrenocorticoids combined with antibiotics and Pep has been derived from experiments with rats (Pliess & Trode 1958, Weller 1955 and 1956). If rats are treated with antibiotics and corticosteroids during a long period of time Pep will often develop. These observations have been interpreted as an indication that PC normally exists as an innocent commensal in the lungs of the rat. When the resistance is diminished as a result of treatment, PC will be able to grow unimpeded and attain a pathogenic character. By analogy the same mechanism may well be of importance in explaining the unusual occurrence of a massive Pep, terminating fatally in our patients. Massive administration of steroids is frequently used in current therapy. On the basis of an ubiquitous occurrence of PC one would therefore expect massive Pep to be common in such cases. Since that is not the case additional, unknown pathogenic factors may also be operative.

In a review of Pep in association with prolonged corticosteroid therapy in older children Kossel (1962) pointed to the fact the Pep cases occurred in connection with viral "grippaler infekt ketten". Although there was no direct proof of a coexisting virus infection in our cases this possibility cannot be wholly eliminated.

In this connection the frequent coexistence of Pep and cytomegalic inclusion body disease may be mentioned (Hamperl 1956, Kramer 1962, Symmers 1960). Signs of the latter disease, however, were not found in our cases.

#### SUMMARY

Two cases of *Pneumocystis pneumonia* are presented, one in a sub-leukemic acute leukemia in a four-year-old girl and one in chronic lymphatic leukemia in a 55-year-old man. The patients were treated with large doses of corticosteroid for prolonged periods, periodically



crescent-formed Gram positive parasites were clearly demonstrated and with the silver methenamine method numerous organisms were also seen (Fig 8). There was a moderate interstitial diffuse fibrosis and here and there broadening of alveolar walls and a moderate infiltration by lymphocytes, histiocytes and some granulocytes. In methyl green pyronine stain only a very small number of plasma cells were seen. There was only a slight leukaemic infiltration, in places with small accumulations of lymphocytes in and around blood vessels.

Examination of the liver and the spleen revealed leukaemic infiltrations with small accumulations of mature lymphocytes in the portal tracts in the liver and in the spleen, a rather diffuse infiltration with lymphocytes.

## DISCUSSION

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In retrospect the respiratory distress during the last days of life in both of our cases taken together with the roentgenological lung picture

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## ACUTE HYPERTENSIVE VASCULAR DISEASE

### 1. Relation between Blood Pressure Changes and Vascular Lesions in Different Forms of Acute Hypertension

By

JØRN GIESE

Received 29.11.64

Acute renal ischaemia in rats has been shown to elicit a syndrome characterized by a leakage of plasma from the intravascular compartment into the tissues and serous cavities together with a deposition of serum proteins in the media of small arteries and arterioles (Giese 1962). Further studies have shown that essentially the same syndrome can be elicited by the administration of rat kidney extracts, purified hog renin or synthetic angiotensin to nephrectomized rats (Giese 1963).

This paper reports studies aiming at a further elucidation of the mechanisms involved in the causation of this type of acute vascular damage and plasma leakage. Firstly, blood pressure recordings have been performed in rats exposed to the various experimental conditions mentioned above. Secondly, the effect of quinine on the blood pressure response induced by acute renal ischaemia was studied; administration of quinine has been shown to suppress the formation of serous effusions and tissue oedema in this experimental situation (Giese 1962). Thirdly, acute hypertension was induced by means of pressor agents other than renin or angiotensin; nephrectomized rats were used in order to exclude any participation of the renal renin system. The blood pressure responses and vascular lesions elicited in this way were studied for comparison with the experiments involving the renin-angiotensin system.

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I wish to thank IFO Pharmaceuticals for supplies of heparin and Burroughs-Wellcome & Co. (represented by Holger Andreasen, Copenhagen) for supplies of methamphetamine.

combined with antibiotics and cytostatics. Clinically and morphologically the leukaemias were in a state of remission at death.

At autopsy bilateral, massive *Pneumocystis pneumonia* was found to be the immediate cause of death in both cases. The possibility that the combination of leukaemia and *Pneumocystis pneumonia* were not fortuitous is discussed. It is suggested that the combination of leukaemia and intense treatment with corticosteroids and cytostatics by lessening the resistance prepared the ground for a massive fatal *Pneumocystis pneumonia*.

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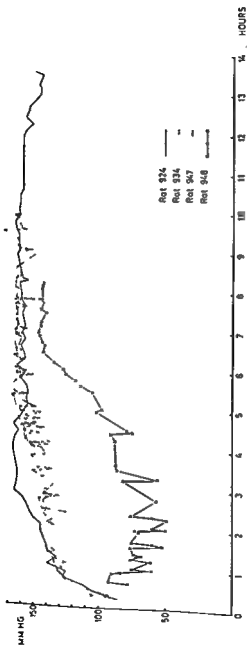


Fig. 1  
Recovery of blood pressure in four rats subjected to application of narrow silver clips  
on both renal arteries

## MATERIAL AND METHODS

**Animals** Female albino rats each weighing approximately 200 grams were used

**Blood pressure recordings** were performed by means of a *Tybjerg Hansen* capacitance manometer, which was connected with a *Varian G-14* recorder. About 30 mm length of polyethylene tubing PI 10 (Clay - Adams) was connected to a 0.5 mm bore polythene cannula (Sterivac Allen & Hanbury Ltd) leading to the transducer. The PI 10 tubing was inserted into the carotid artery and advanced towards the aorta. Heparin saline (25 i.u. per ml) was used for filling the catheters and for flushing. Electrical damping was mostly employed in order to obtain recordings of mean pressure. Calibration pressures (0 and 180 mm Hg) were applied to the transducer approximately every 20 minutes.

**Induction of acute kidney ischaemia** Clamps made of silver wire internal width 0.15 mm were applied on both renal arteries.

**Preparation of rat kidney extract** Normal rat kidneys were homogenized in 4 ml of ice cold saline per gram of kidney tissue in a glass tissue grinder. The homogenate was left for three days at 4° C for extraction. After centrifugation dialysis against normal saline was performed in the cold. The extract was stored at about -18° C and recentrifuged after thawing before use. The final dilution was such that either 4 or 10 ml would contain the extractable non dialyzable substances from 1 gram of kidney tissue.

**Angiotensin** Hypertensin GIBA (val<sup>5</sup> angiotensin II amide) was dissolved in normal saline to a concentration of 50 micrograms per ml.

**Noradrenaline** a solution containing 0.2 mg of noradrenaline bitartrate (corresponding to 0.1 mg of noradrenaline) per ml was used.

**Methoramine** ( $\beta$  hydroxy  $\beta$  (2,5 dimethoxyphenyl) isopropylamine) was used as a solution of methoramine hydrochloride containing 20 mg per ml (Vasoxine, Burroughs Wellcome & Co.)

**Quinine** was used as a 10 per cent solution of quinine hydrochloride dissolved in a 10 per cent solution of urethane.

an automatic infusion machine  
used in the experiments (clamping of renal  
arteries) were performed under ether anaesthesia.

After performance of nephrectomy or clamping of the renal arteries a subcutaneous injection of diethylbarbituric acid (30 mg of the sodium salt (barbital sodium)) was given. This drug is excreted primarily through the kidneys and a very prolonged action of this single dose was evident in rats subjected to nephrectomy or kidney ischaemia, thus making prolonged recording of blood pressure manageable without restraint.

**Histological technique** The tissues were fixed in Bouin's fluid. Periodic acid Schiff staining (PAS) was used as a routine. In some cases Mallory's phosphotungstic acid haematoxylin stain for fibrin (P1 AH) and Masson's trichrome stain in *Goldner's* (1938) modification was used.

**General remarks** The duration of the experiments was generally about 24 hours except where otherwise stated.

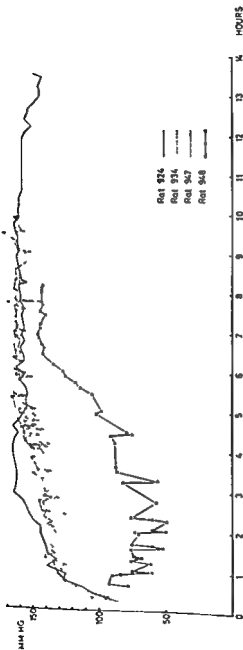
In the blood pressure records reproduced in this paper zero time is taken as the time at which nephrectomy was completed or (figs 1 and 4) clamping of both renal arteries had been performed.

## RESULTS

### *A Studies on the Blood Pressure During the Development of Acute Vascular Disease*

#### *1 Acute Renal Ischaemia Induced by Bilateral Application of Narrow Silver Clips on the Renal Arteries*

This procedure was performed in four rats, the blood pressure was recorded for 8-14 hours after the operation as shown in Fig. 1. A pronounced rise of the blood pressure was observed. In three experiments the pressure rose to 150-160 mm Hg during the first four hours.



*Fig. f*  
Rectals of blood pressure in four rats subjected to application of narrow silver clips  
on both renal arteries

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*Quinine* was used as a 10 per cent solution of quinine hydrochloride dissolved in a 10 per cent solution of urethane.

*Intravenous infusions* were given by an automatic infusion machine.

*Anaesthesia.* The operations involved in the experiments (clamping of renal arteries, nephrectomy, insertion of catheters) were performed under ether anaesthesia. After performance of nephrectomy or clamping of the renal arteries a subcutaneous injection of diethylbarbituric acid (30 mg of the sodium salt (barbital sodium)) was given, this drug is excreted primarily through the kidneys and a very prolonged action of this single dose was evident in rats subjected to nephrectomy or kidney ischaemia thus making prolonged recording of blood pressure manageable without restraint.

*Histological technique.* The tissues were fixed in Bouin's fluid. Periodic acid Schiff staining (PAS) was used as a routine. In some cases Mallory's phosphotungstic acid haematoxylin stain for fibrin (PTAH) and Masson's trichrome-stain in Goldner's (1938) modification was used.

*General remarks.* The duration of the experiments was generally about 24 hours except where otherwise stated.

In the blood pressure records reproduced in this paper, zero time is taken as the time at which nephrectomy was completed or (figs. 1 and 4) clamping of both renal arteries had been performed.

## RESULTS

### A Studies on the Blood Pressure During the Development of Acute Vascular Disease

#### 1. Acute Renal Ischaemia Induced by Bilateral Application of Narrow Silver Clips on the Renal Arteries

This procedure was performed in four rats, the blood pressure was recorded for 8-14 hours after the operation as shown in Fig. 1. A pronounced rise of the blood pressure was observed, in three experiments the pressure rose to 150-160 mm Hg during the first four hours.

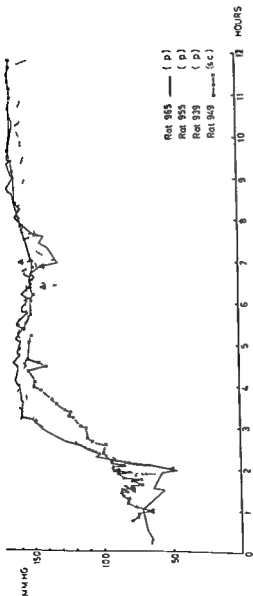


Fig. 2  
 Blood pressure changes in four nephrectomized rats after intraperitoneal (i p) or subcutaneous (s c) injections of rat kidney extract given at the times indicated by black triangles



and remained at this level for the rest of the period of observation. In one rat (No 948) the pressure rise occurred much later, between the fifth and seventh hour, a level around 145 mm Hg being attained. In two rats it was possible to resume the recording 22 to 25 hours after the operation. One of these (No 934) showed a blood pressure slowly decreasing from 160 to 135 mm Hg over four hours, the other (No 924) had a blood pressure in the range 110-120 mm Hg.

Autopsy was performed 21-27 hours after the operation. A more or less complete infarction of both kidneys was observed. Signs of plasma leakage (oedema of mesentery and pancreas and small serous effusions) were found in all cases. Microscopy showed the presence of PAS positive deposits in pancreatic or intestinal arterial vessels.

## 2 *Administration of Rat Kidney Extract to Nephrectomized Rats*

In three experiments two intraperitoneal injections of rat kidney extract were given at  $1\frac{1}{2}$ -2 hours and  $6\frac{1}{2}$ -7 hours after nephrectomy. The volume injected was 5 ml containing extractable substances from 0.5 grams of normal rat kidney. A rapid rise of the blood pressure was observed as demonstrated in Fig. 2. Levels about 140 mm Hg after one hour and about 150-160 mm Hg after two hours were found, the pressure staying at this level (150-160 mm Hg) for the remaining period of observation. The blood pressure was recorded for 8-12 hours.

In one experiment (rat 949) extractable substances from 1 gram of kidney tissue, contained in 4 ml of fluid, were administered subcutaneously (multiple injection sites). The rise of blood pressure was somewhat slower, attaining finally approximately the same high level.

Autopsy showed the previously described syndrome of plasma leakage and acute vascular disease.

## 3 *Continuous Infusion of Synthetic Angiotensin into Nephrectomized Rats*

The dosage of angiotensin was approximately 17 micrograms per kg per min. Three experiments were performed (Fig. 3); recording trouble has interrupted one of the records. The blood pressure rises at once at the start of the infusion to very high levels. Later on the blood pressure stabilizes at different levels which are maintained for the period of observation. The pressures recorded the next morning after about 21 hours of continuous infusion were about 105 mm Hg (rat 954), 125 mm Hg (rat 956) and 130 mm Hg (rat 1109).

Severe degrees of pancreatic and mesenteric oedema and typical vascular lesions with PAS positive deposits were found at autopsy.



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## 2 *Administration of Rat Kidney Extract to Nephrectomized Rats*

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In one experiment (rat 949) extractable substances from 1 gram of kidney tissue, contained in 4 ml of fluid, were administered subcutaneously (multiple injection sites). The rise of blood pressure was somewhat slower, attaining finally approximately the same high level.

Autopsy showed the previously described syndrome of plasma leakage and acute vascular disease.

## 3 *Continuous Infusion of Synthetic Angiotensin into Nephrectomized Rats*

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Severe degrees of pancreatic and mesenteric oedema and typical vascular lesions with PAS-positive deposits were found at autopsy.

## B *The Effect of Quinine on the Blood Pressure Response Following Bilateral Clamping of the Renal Arteries*

Since it has been shown in an earlier paper (Giese 1962), that administration of quinine will suppress at least the macroscopic manifestations of plasma leakage after the induction of bilateral kidney ischaemia it seemed indicated to study the effect of quinine on the blood pressure response in this experimental situation.

Three rats were subjected to bilateral application of 0.15 mm silver clips on the renal arteries. Quinine hydrochloride was administered in the dosage employed in the earlier experiments (two subcutaneous injections were given at one and six hours after the operation, respectively dosage 125 mg per kilogram of body weight given in each of the injections).

The recordings are depicted in Fig. 4 they should be compared with the recordings in Fig. 1. It is clearly seen that the blood pressure level is rather low in rats 1028 and 1029 not exceeding 120–125 mm Hg during ten hours of recording. Rat 1003 shows a higher pressure level although never exceeding 140 mm Hg. These results thus demonstrate that quinine in the dosage employed partly or totally prevents the development of high blood pressure after the induction of bilateral kidney ischaemia.

Autopsy after about 24 hours showed no effusions or tissue oedema. Microscopy showed no vascular lesions in rat 1028 and a few lesions in rats 1029 and 1003.

## C *A Study on Morphological Changes in the Vascular System of the Rat Occurring After Induction of Acute Hypertension by means of Sympathomimetic Agents*

The three types of acute hypertension studied in part A of this paper are all linked to the renin-angiotensin system. It now seemed of interest to study the effects of acute hypertension induced by means of other pressor agents in order to exclude any participation of the renal renin system. The experiments were performed in nephrectomized rats. Unfortunately the sustained hypertension observed in the experiments involving renin or angiotensin could not be duplicated by the administration of noradrenaline or methoxamine in the dosages employed. Still some information was gained from these experiments.

### 1 *Infusions of Noradrenaline (about 3–4 Micrograms per kg per min)*

In two experiments the infusion was given over approximately ten hours and the blood pressure was recorded during this period. In a further five rats the infusion was continued for about 22 hours; in three of these the blood pressure was recorded during the first five to

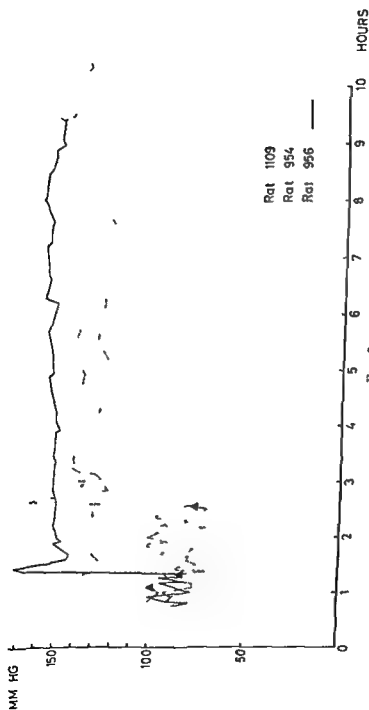


Fig. 3  
 Blood pressure in three nephrectomized rats receiving a continuous infusion of synthetic angiotensin (about 17 micrograms per minute). Black triangles signal the start of infusion.

## B *The Effect of Quinine on the Blood Pressure Response Following Bilateral Clamping of the Renal Arteries*

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The recordings are depicted in Fig. 4, they should be compared with the recordings in Fig. 1. It is clearly seen, that the blood pressure level is rather low in rats 1028 and 1029, not exceeding 120–125 mm Hg during ten hours of recording. Rat 1005 shows a higher pressure level, although never exceeding 140 mm Hg. These results thus demonstrate, that quinine in the dosage employed partly or totally prevents the development of high blood pressure after the induction of bilateral kidney ischaemia.

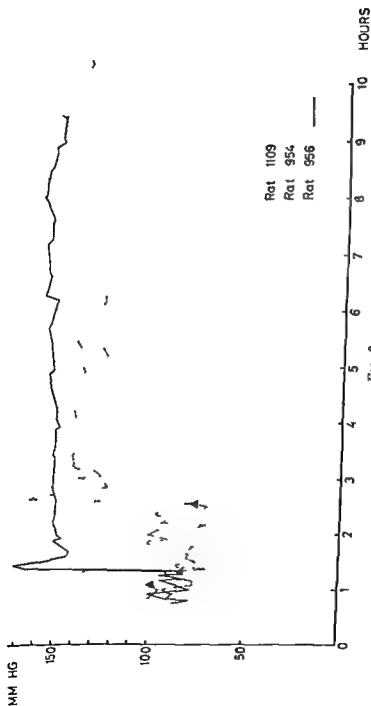
Autopsy after about 24 hours showed no effusions or tissue oedema. Microscopy showed no vascular lesions in rat 1028 and a few lesions in rats 1029 and 1005.

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*Fig 3*  
 Blood pressures in three nephrectomized rats receiving a continuous infusion of synthetic angiotensin (at out 17 micrigrams per liter min)  
 Black triangles signal the start of infusion

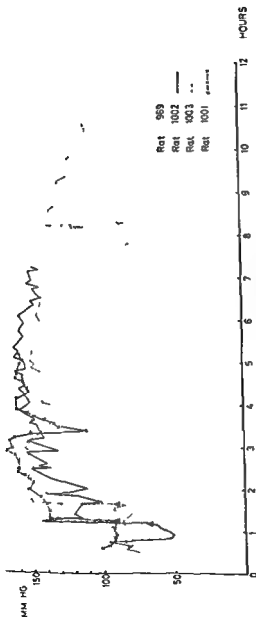
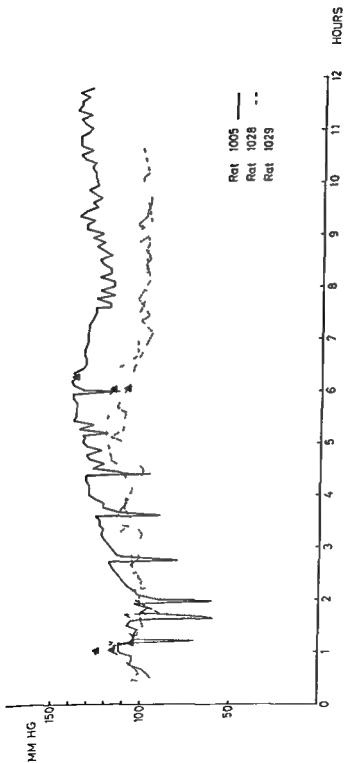


Fig 5  
Records of blood pressure in four nephrectomized rats, receiving noradrenaline (about 3-4 micrograms per kg per min) as a continuous infusion. Black triangles signal the start of infusion.





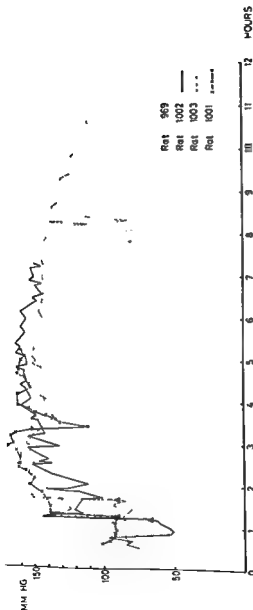


Fig. 5

Records of blood pressure in four nephrectomized rats receiving noradrenaline (about 3.4 micrograms per kg per min) as a continuous infusion. Black triangles signal the start of infusion.

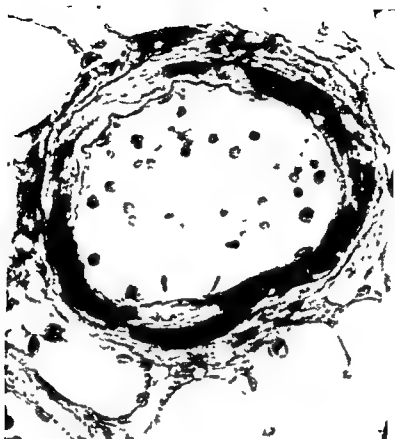


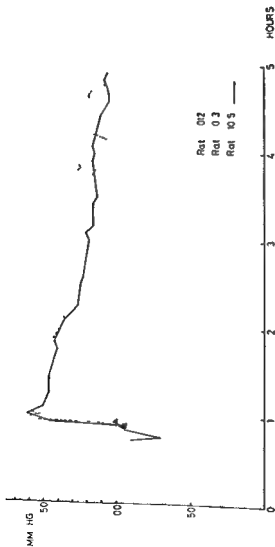
Fig 6

Rat 1001 Nephrectomy—infusion of noradrenaline Mesenteric artery showing fibrin-like deposits in the media Phosphotungstic acid—haematoxylin ( $\times 720$ )

seven hours and again for a short period the next morning Autopsy was performed shortly after terminating the infusion

Recordings from four experiments are given in Fig 5 The blood pressure rises immediately at the start of the infusion, but the subsequent course is variable In some cases the pressure is fairly high for several hours (rats 969—1001—1002), in others the pressure declines (rat 1003) The pressure recorded after about 22 hours of infusion in three rats was in the range 80–100 mm Hg At the termination of the infusion a steep fall in pressure occurred

None of the rats showed the autopsy findings characteristic of renin- or angiotensin-induced vascular disease with plasma leakage, i.e. generalized succulence of the abdominal organs, but slight degrees of localized mesenteric or pancreatic oedema were encountered in some of the rats In five of the rats no or only few vascular lesions were found, but two rats (No 969 and 1001, infused for 10 and 22 hours respectively) showed a fair number of PAS-positive deposits in arteries localized to the mesenteric border of the small intestine Deposits showing fibrin-like staining (dark blue) with Mallory's phosphotungstic acid—haema-



Records of blood pressure in three different mice rats receiving methoxaml a (115-140 micrograms per kg per min) as a continuous infusion is indicated in Black triangles signal the start of infusion

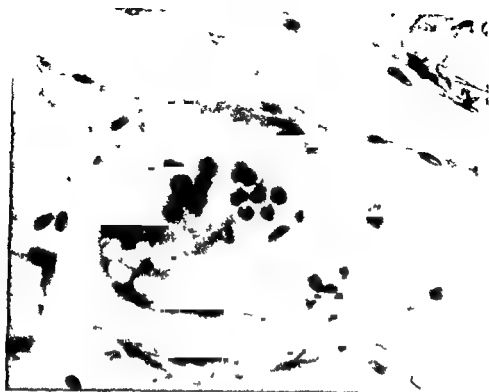


Fig. 8

Rat 1012 Nephrectomy—infusion of methoramine Mesenteric artery showing necrotic PAS positive media PAS stain ( $\times 720$ )

toxylin procedure were demonstrable in a number of the damaged arteries (Fig. 6). Small (rat 969) or quite substantial (rat 1001) such sinophilic deposits were found in some of the damaged arteries using Goldner's modification of Masson's trichrome stain. Some of the vessels showing deposits were necrotic, being devoid of nuclei in the PAS positive parts of the vessel walls.

## 2. Infusions of Methoramine (115-140 Micrograms per kg per min.)

The dose level selected for these experiments proved to be rather toxic to the rats and 24 hour infusions were difficult to perform. Accordingly only short term infusions were performed lasting four to five hours. Five rats were infused, one unanesthetized, four under light barbitol anesthesia. In three rats the blood pressure was recorded during the experiment (Fig. 7). A very high pressure is obtained initially, but the pressure decreases more or less rapidly.

The most interesting autopsy finding was the presence of a pronounced oedema of the mesentery in four of the rats, two of these showed a moderate degree of pancreatic oedema. In these four rats vascular lesions were found in arteries located at the mesenteric border of the small intestine. PAS positive deposits were found in the media

many of these small arteries were severely damaged, showing a homogeneous PAS-positive media with a few distorted nuclear remnants (Fig 8) No deposits could be demonstrated with the PTAH-stain or the trichrome-method The damaged vessels appeared much dilated in the sections

## DISCUSSION

In the previous papers (Giese 1962 and 1963) forming the background of the present report, the development of plasma leakage and plasma protein deposits in the arterial walls was tentatively considered as a phenomenon indicating a general increase of endothelial permeability, occurring under circumstances combining the presence of renal insufficiency and the introduction of large amounts of renin into the circulation Asscher & Anson (1963) likewise considered the manifestations encountered after administration of rat kidney extract to nephrectomized rats to be caused by a permeability factor contained in the extract

The present experiments were performed in order to obtain more information concerning the relative importance of pressor effects and possible permeability-increasing effects of renin and angiotensin

The blood pressure recordings described in the first part of the paper show, that the application of narrow silver clips on both renal arteries is followed by the rapid development of acute hypertension The mean pressure rises to around 145-160 mm Hg and remains at this level for many hours Approximately the same level of sustained high blood pressure is attained when renin-containing rat kidney extract is administered to nephrectomized rats Continuous infusion of angiotensin into nephrectomized rats likewise induces a sustained elevation of blood pressure, although sometimes at a somewhat lower level In the few cases, in which the pressure recording could be resumed the next morning, the pressures were varying, sometimes still elevated, sometimes not This was not unexpected since the leakage of plasma from the vascular system must tend to lower the pressure All of the experimental animals showed autopsy findings indicating plasma leakage such as oedema of the mesentery and pancreas, and vascular lesions with PAS-positive deposits were found in the walls of pancreatic and mesenteric small arteries

It was known from previous studies (Giese 1962) that administration of quinine to rats exposed to acute bilateral kidney ischaemia would suppress the macroscopic manifestations of plasma leakage (tissue oedema and serous effusions) to a high degree These experiments were originally inspired by reports on the effectiveness of quinine as an 'anti permeability agent' in experimental conditions involving increased capillary permeability (Carone & Spector 1960, Spector & W. Houghby 1960) The blood pressure recordings under quinine treatment show, that a more or less pronounced hypotensive effect of

quinine in the dosage employed is quite evident. This effect can be due to a myocardial or a direct vasodilator action of quinine. Although these experiments do not exclude, that an "anti-permeability effect" of quinine can be of importance, they indicate that the suppression of pathologic manifestations may be due to the effect of quinine on the blood pressure.

The effects of long-term infusions of *noradrenaline* have been studied in rabbits by *Blacket, Pickering & Wilson* (1950) and *Pugh, Pickering & Blacket* (1952). The initial hypertension was not maintained, and no vascular lesions were found. In the present studies significant mesenteric arterial lesions were found in only two out of seven nephrectomized rats infused for 10–22 hours, but the lesions encountered show histological characteristics resembling renin- or angiotensin-induced vascular damage, including the presence of PAS-positive and fibrin-like deposits in the media.

The induction of arterial necrosis by *methoxamine* was first described by *Herbertson & Kellaway* (1960), they described the frequent occurrence of pancreatic oedema and mentioned that fibrin-like material is found only occasionally in very small amounts in the necrotic arteries. The present studies are in accordance with the original description, it is shown that the arterial lesions can develop in nephrectomized rats and the presence of—often severe—mesenteric oedema is noted. It seems noteworthy, that oedema of the pancreas or mesentery has been found in several other experimental situations, all involving the presence of high blood pressure in rats with hypertensive encephalopathy (*Byrom* 1954), in rats subjected to bilateral renal ischaemia with infarction (*Giese* 1962) and in nephrectomized rats treated with rat kidney extract, hog renin or synthetic angiotensin (*Giese* 1963). The arterial lesions caused by methoxamine differ somewhat from the lesions induced by renin or angiotensin, no fibrin-like deposits can be identified by the phosphotungstic acid – haematoxylin stain. In evaluating the vascular lesions it must be remembered, that the blood pressure decreases rather rapidly in this type of acute hypertension.

The experiments reported in this paper cannot offer a final answer to the question, whether the renin-angiotensin system—under the experimental conditions described—induces an exudation of plasma into the serous cavities, the abdominal tissues and the arterial walls by way of the pressor effect or by way of a (hypothetical) permeability-increasing effect on the vascular endothelium. But considered together these experiments seem to support the first possibility. A pronounced and long maintained hypertension is the common finding in the three experimental situations studied in part A of this paper. The effects of quinine on the pathological manifestations might be explained by the effect on the blood pressure. The very acute occurrence of severe mesenteric or pancreatic oedema in methoxamine induced hypertension under circumstances where any participation of the renal renin system has

been excluded, would point to the prime importance of high intravascular pressure in the genesis of this manifestation of plasma leakage. Likewise, the occasional finding of "fibrinoid" deposits in arterial walls after infusion of noradrenaline into nephrectomized rats tends to incriminate high blood pressure as the main responsible factor.

### SUMMARY

1. Blood pressure recordings for seven to twelve hours after the induction of severe renal ischaemia, after the administration of rat kidney extract to nephrectomized rats or during the continuous infusion of synthetic angiotensin into nephrectomized rats have demonstrated the occurrence of severe and sustained hypertension in all of the three experimental situations. Acute vascular disease develops rapidly under these circumstances together with other signs of leakage of plasma from the intravascular compartment.

2. Administration of quinine, which suppresses manifestations of plasma leakage after induction of bilateral kidney ischaemia, decreases the level of blood pressure in this experimental condition.

3. Continuous infusion of noradrenaline into nephrectomized rats induces an acute hypertension of a varying stability. Arterial necrosis with PAS positive and fibrin like deposits in the media was found in two out of seven rats after 10 to 22 hours of infusion.

4. Infusion of methoxamine into nephrectomized rats induces a high blood pressure initially, but the pressure declines rather rapidly. The severe vascular lesions encountered are described, signs of plasma leakage are frequently present.

5. The experiments point to the prime importance of high intravascular pressure in the genesis of vascular lesions and plasma leakage.

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## ACUTE HYPERTENSIVE VASCULAR DISEASE

■ *Studies on Vascular Reaction Patterns and Permeability Changes  
by means of Vital Microscopy and Colloidal Tracer Technique*

By

JØRN GIESP

Received 29.11.64

In 1961 Wajno, Palade & Schoeffl published a study on chemical inflammation based on a method referred to as "vascular labelling", permitting the identification of leaking vessels by means of visible accumulations of foreign particles within their walls. By the local injection of histamine or serotonin an increased exudation of plasma from the small vessels (in case venules) is induced, if the plasma has been previously loaded with colloidal particles of carbon, these particles will be retained and accumulate within the wall of the leaking vessel, being too large to pass through the basement membrane. Time is allowed for clearing of the blood stream by the reticulo endothelial system so that no carbon particles remain in the blood at the time of sacrifice. The leaking vessels are thus outlined in black—a "vascular labelling" takes place.

It was reasoned that since earlier studies (Giese 1962 and 1963) have shown an increased passage of fluid and plasma proteins through the vascular walls to be an essential step in the pathogenesis of acute hypertensive vascular disease, it ought to be possible to identify leaking arteries, arterioles and smaller vessels at the same time and at an early stage of the disease by means of this technique. This proved to be the case.

Next this "vascular labelling" technique was used in combination with vital microscopy in order to study the process of labelling directly during its development *in vivo*. This part of the study gave clear evidence concerning the relationship between the vascular reaction patterns observed after induction of acute hypertension and the occurrence of focal increases of vascular permeability.

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University Institute of Pathological  
With permission to use the ultramicrotome in his department I thank Dr  
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## ACUTE HYPERTENSIVE VASCULAR DISEASE

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Received 29.1.64

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Th. —

## MATERIAL AND METHODS

**Animals** Female albino rats each weighing about 200 grams were used

**Blood pressure recordings** and preparation of rat kidney extracts were performed as described in the preceding paper

**Angiotensin** Hypertensin CIBA (val<sup>1</sup> angiotensin II-amide) was dissolved in physiological saline. Generally a concentration of 50 micrograms per ml was used, in a few experiments (see Results part B, section 4) concentrations of 3 M or 18 micrograms per ml were used

**Noradrenaline** a solution containing either 0.2 or 2 mg of noradrenaline bitartrate corresponding to 0.1 or 1 mg of noradrenaline per ml was used

**Methoxamine** was used as a solution of methoxamine hydrochloride containing 20 mg per ml ('Vasoxine', Burroughs Wellcome & Co.)

**Hydralazine** Aprisolin CIBA was used as a solution containing 20 mg per ml

**Dihydralazine** Nepresol CIBA was used as a solution containing 1 mg per ml

**Colloidal carbon** (carbon black especially prepared for experimental use (Guenther Wagner Pelikan Werke Hannover Germany, batch C 11/1431 a)) This preparation is the same as used by Majno Palade & Schoeffl (1961), according to these authors it contains about 100 mg/g of carbon with an indicated average particle size of 200 Å, it is stabilized with 4.5 per cent fish glue and contains 1.3 per cent phenol as a preserving agent

**Intravenous infusions** were given by an automatic infusion machine

**Anaesthesia** The operations involved in the experiments (nephrectomy, insertion of catheters, incision of abdominal windows) were performed under ether anaesthesia. After completion of nephrectomy or (when intact rats were used) at the beginning of the operation a subcutaneous injection of diethylbarbituric acid (30 or 40 mg of the sodium salt (barbital sodium)) was given, the higher dose being used in the experiments involving vital microscopy. In these experiments additional doses of barbital sodium (about 10 mg) were given intravenously when required at later stages of the experiment

**Vital microscopy** A very simple abdominal window was constructed based on parts of the more elaborate equipment described by Palmer & Wilkins (1960). A ring of plexiglas (external diameter 40 mm, internal diameter 28 mm, height 9 mm) was cemented to a circular plate of the same material (diameter 40 mm, thickness 2 mm). The ring had an excavation around the whole circumference and was fitted with a plexiglas rod which could be fixed in a laboratory stand. As described by Palmer & Wilkins the insertion of such a window is very simple, the abdomen is opened in the midline and the ring is inserted so that the edges of the abdominal muscles fit into the excavation like the tyre on the rim of a wheel. The muscular edges are firmly fixed in this position by a thread encircling the excavation of the ring. Incident light from a microscopy lamp was used. Observations were carried out

Electronic flash was used for photography

in was used for fixation. Clearing of tissues

stereomicroscope was performed by means

of methyl benzoate and oil of anise after dehydration in alcohols

In order to preserve the *in vivo* state of constrictions and dilatations in intestinal arteries for demonstration in a fixed preparation it was tried to perform instantaneous freezing of intestinal loops by decanting a freezing mixture (acetone/solid carbon dioxide) into the abdominal cavity of a deeply anaesthetized rat receiving a continuous infusion of angiotensin. The frozen loops were removed and placed in absolute alcohol at  $-18^{\circ}\text{C}$  for about one week to permit freeze substitution afterwards they were cleared by the usual method

For the preparation of thin sections tissue was embedded in Vestopal W after fixation in formalin and dehydration in acetone. 1 micron sections were cut on a LKB ultramicrotome and affixed to glass slides coated with albumin glycerol. Staining was performed with a 2 per cent aqueous toluidine blue solution

**General remarks** In all experiments polyethylene catheters were placed in the carotid artery (for blood pressure recording) in the jugular vein (for intravenous infusion of pressor agents) and in the femoral vein (for administration of colloidal carbon)

I wish to thank CIBA Pharmaceuticals Copenhagen for supplies of Aprisolin and Nepresol

## RESULTS

*A Demonstration of Increased Vascular Permeability During Acute Hypertension Induced by Synthetic Angiotensin*

Infusion of synthetic angiotensin into the nephrectomized rat has been shown to produce acute vascular disease with great regularity when suitable doses are employed (Giese 1963)

Six experiments were performed. About one hour after nephrectomy an infusion of synthetic angiotensin (about 1.7 micrograms per kg per min) into the jugular vein was started. Three hours later an injection of 0.2 ml of colloidal carbon was given through the femoral catheter without interrupting the angiotensin infusion which was continued for another hour after which the rat was decapitated under ether anaesthesia. After centrifugation of the blood the serum did not contain visible carbon.

At autopsy vascular labelling was macroscopically visible in five of the six rats. Small arteries at the mesenteric border of the small intestine and at the intestinal surface were labelled in black. In relation to these labelled arteries areas showing more diffuse deposits were found suggesting a labelling of smaller vessels. When further studied by transillumination under a stereomicroscope (after fixation and clearing of the intestinal loops) the carbon deposits in the arteries were found to be localized in the media of the vessel arranged as parallel streaks in the vessel wall perpendicular to the long axis of the artery (Fig. 1) often forming a ring like pattern encircling the artery (Fig. 2). The deposits were found either as a more continuous investment for a variable distance or as focal cuffs around the artery. A site of predilection was the mesenteric border of the small intestine but "cuff like" deposits were often found in the course of the artery over the surface of the intestine. In these preparations the labelled parts of the arteries always appeared dark.

Thin sections of the arteries showed that the carbon particles were located in some places in the intima and in others in the media. The particles corresponded to the intercellular areas between the smooth muscle cells of the media. More massive deposits of carbon were also found but it was difficult to make out the exact localization of these whether intra- or extracellular (Fig. 3). In some places carbon particles were found on either side of the internal elastic membrane the relationship of the lumenally situated particles to the endothelial cytoplasm could not be made out.

The areas showing diffuse deposits indicating abnormal leakiness of smaller vessels were predominantly located in close relation to labelled arteries. In some cases the diffuse deposits were in relation to an area of diffuse thickening of the type of atherosclerosis. In some cases (venules?) it was not possible to determine the location of carbon deposits in these small vessels.

## MATERIAL AND METHODS

**Animals** Female albino rats, each weighing about 200 grams were used

**Blood pressure recordings** and preparation of rat kidney extracts were performed as described in the preceding paper

**Angiotensin** Hypertensin CIBA (val<sup>1</sup> angiotensin II amide) was dissolved in physiological saline. Generally a concentration of 50 micrograms per ml was used in a few experiments (see Results, part II section 4) concentrations of 3.9 or 18 micrograms per ml were used

**Noradrenaline** a solution containing either 0.2 or 2 mg of noradrenaline bitartrate, corresponding to 0.1 or 1 mg of noradrenaline per ml was used

**Methoxamine** was used as a solution of methoxamine hydrochloride containing 20 mg per ml (Vasoxine, Burroughs Wellecome & Co)

**Hydralazine** Apresolin CIBA was used as a solution containing 20 mg per ml

**Colloidal carbon** (carbon black especially prepared for experimental use (Guenther-Wagner Pelikan-Werke Hannover, Germany, batch C 11/1431 a) This preparation is the same as used by Wajno Palade & Schoeffl (1961), according to these authors it contains about 100 mg/cc of carbon with an indicated average particle size of 200 Å, it is stabilized with 4.5 per cent fish glue and contains 1.3 per cent phenol as a preserving agent

**Intravenous infusions** were given by an automatic infusion machine

**Anaesthesia** The operations involved in the experiments (nephrectomy insertion of catheters insertion of abdominal windows) were performed under ether anaesthesia. After completion of nephrectomy or (when intact rats were used) at the beginning of the operation a subcutaneous injection of diethylbarbituric acid (30 or 40 mg of the sodium salt (barbital sodium)) was given the higher dose being used in the experiments involving vital microscopy. In these experiments additional doses of barbital sodium (about 10 mg) were given intravenously when required at later stages of the experiment

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out of methyl benzoate and oil of anise after dehydration in alcohols

In order to preserve the *in vivo* state of constrictions and dilatations in intestinal arteries for demonstration in a fixed preparation it was tried to perform instantaneous freezing of intestinal loops by decanting a freezing mixture (acetone/solid carbon dioxide) into the abdominal cavity of a deeply anaesthetized rat receiving a continuous infusion of angiotensin. The frozen loops were removed and placed in absolute alcohol at  $-18^{\circ}\text{C}$  for about one week to permit freeze substitution after which they were cleared by the usual method

For the preparation of thin sections tissue was embedded in Vestopal W after fixation in formalin and dehydration in acetone. 1 micron sections were cut on a

with albumin glycerol stain  
in blue solution

carotid artery (for blood pressure measurements) and in the femoral vein (for intravenous infusion of pressor agents) and in the femoral vein (for administration of colloidal carbon)

I wish to thank CIBA Pharmaceuticals (Copenhagen) for supplies of Apresolin and Nepresol



Figs 3 &amp; 4

- Fig 3 Rat 1033 Nephrectomy—angiotensin infusion Formalin fixation embedding in Vestopal W 1 micron section Intestinal artery showing deposits of carbon in the media Some of the discrete deposits are located in the intercellular areas between the smooth muscle cells More massive deposits are also seen the localization of these is uncertain Toluidine blue stain ( $\times 1800$ )
- Fig 4 Rat 1033 Nephrectomy—angiotensin infusion Preparation as in Fig 3 Section from the intestinal wall showing a small vessel containing carbon in the wall Toluidine blue stain  $\times 1800$





Figs 1 2

- Fig 1* Rat 1033 Nephrectomy—angiotensin infusion Formalin fixed and cleared preparation Mesenteric artery showing heavy deposits of carbon in the wall arranged in streaks perpendicular to the long axis of the vessel ( $\times 184$ )
- Fig 2* Rat 1033 Nephrectomy—angiotensin infusion Preparation as in Fig 1 Arteries at the mesenteric border of the small intestine demonstrating pronounced carbon deposits in ring like arrangements around the circumference of the vessels A labelled dilated branch leads to an area of heavy diffuse labelling ( $\times 46$ )

was hard to make out in paraffin sections in a limited number of thin Vestopal sections occasional small vessels were observed showing a deposition of carbon in the wall (Fig. 4)

Since the continuous measurement of the blood pressure necessitates a varying number of flushings with saline through the carotid catheter in order to keep it patent and since these injections of saline into the arterial system might possibly be of importance the experiment was repeated in five rats treated in exactly the same way except that the carotid artery was not cannulated and no blood pressure recording was performed. Of these five rats four showed deposits of carbon in intestinal arteries and diffuse deposits in smaller vessels.

The experiments were further repeated in five nephrectomized rats receiving an infusion of physiological saline instead of angiotensin. The blood pressure was around 100 mm Hg in four of these rats no arterial deposits of carbon and only few areas showing deposits in minute vessels were found in two of the rats the endothelial surface of a few small veins was coated with carbon. One rat had an unusually low blood pressure (around 60-70 mm Hg) and showed several areas of diffuse labelling together with coating of the endothelial surface of a few small veins with carbon. These changes were confined to a small section (length about 6 cm) of the intestine. No arterial deposits were found.

Blood pressure recordings from two angiotensin experiments and one control experiment are shown in Fig. 5.

## *B Vital Microscopy of Intestinal Arteries During Acute Hypertension Vascular Reaction Patterns and Permeability Changes*

The use of an abdominal window offered excellent possibilities for microscopical observations during several hours. Several intestinal arteries can be studied in the field of vision and no signs of damage to the intestines have been observed.

A number of experimental situations were studied by the combination of vital microscopy and the vascular labelling technique.

### *1 Infusion of Angiotensin (about 17 Micrograms per kg per min) into Nephrectomized Rats*

This method for induction of acute hypertension was chosen for primary studies in order to obtain more information concerning the mechanisms involved in the development of the permeability changes described in part A of this paper.

Fourteen experiments were performed generally the infusion of angiotensin was started one to two hours after nephrectomy when the catheters and the abdominal window had been inserted. The blood pressure was measured continuously.

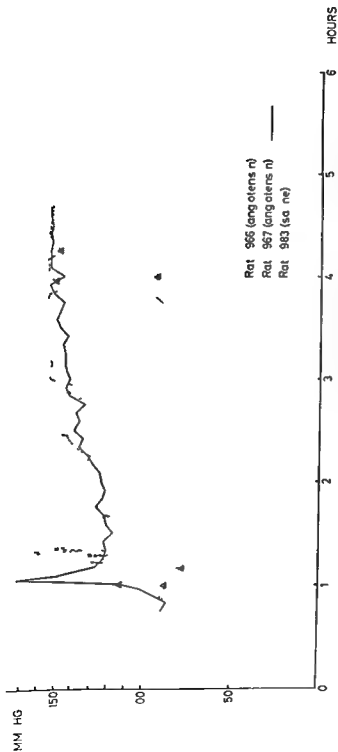
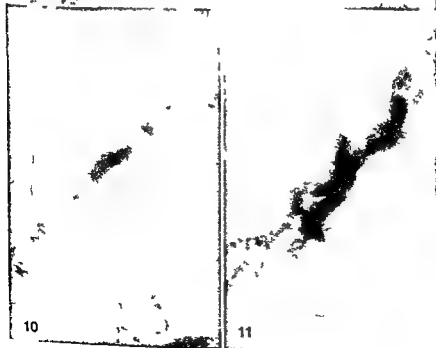


Fig. 5  
Records of blood pressure in two angiotensin experiments and one control experiment. White triangles start of infusions on black triangles injection of colloidal carbon. See text for further explanation.





Figs 6-7

- Fig 6** Rat 1047 Nephrectomy—angiotensin infusion Intestinal loop prepared by immediate freezing—freeze substitution in alcohol—clearing The characteristic pattern of dilations (one fusiform one located in a bifurcation) with intervening constricted parts in the course of the artery is seen ( $\times 45$ )
- Fig 7** Rat 1047 Nephrectomy—angiotensin infusion Another region of the preparation used in Fig 6 Several dilations are seen including dilated parts of the mesenteric arterial arcade separated by constricted segments ( $\times 45$ )

Figs 8-11

- Fig 8** Rat 1070 Intact kidneys—angiotensin infusion Photograph taken *in vivo* through the stereomicroscope Two pronounced dilations in the region of the artery close to the mesentery are separated by a short zone of constriction From the distal dilated segment a side branch goes off, showing dilatation tapering into a severely constricted part ( $\times 60$ )
- Fig 9** Rat 1078 Intact kidneys—angiotensin infusion *In vivo* photograph Intestinal artery showing moderately dilated region with several carbon deposits in the lower part of the picture The left branch is dilated and labelled in the first part thereafter constricted the right branch shows some labelled regions along the course ( $\times 60$ )
- Fig 10** Rat 1078 Intact kidneys—angiotensin infusion *In vivo* photograph Small artery showing labelled regions which are moderately dilated The intervening constricted parts had begun to relax at the time of photography ( $\times 60$ )
- Fig 11** Rat 1078 Intact kidneys—angiotensin infusion *In vivo* photograph Intestinal artery showing several carbon deposits The heavy deposit in the proximal dilated part of the side branch going off towards right is especially noteworthy, since the first deposit in this region was observed 50 seconds after the start of the angiotensin infusion carbon having been injected just before the start of the infusion ( $\times 60$ )



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Fig 12

Rat 1047 Nephrectomy—angiotensin infusion Detail from Fig 11 A single streak like deposit of carbon particles in the wall of a dilated region in an intestinal artery ( $\times 175$ )

In twelve of the experiments a very characteristic pattern of alternating constrictions and dilatations along the course of the intestinal arteries developed immediately at the start of the angiotensin-infusion, simultaneously with the pronounced rise of blood pressure (Figs 6 and 7) The proximal parts of the visible course of the large and small arteriae rectae, i.e. the region close to the mesenteric border of the intestine, were the seat of often pronounced dilatation sometimes two dilatations, separated by a short constricted zone, could be observed (Fig 8) Dilatations, separated by severely constricted segments, were seen in the course of the artery distally towards the antimesenteric border (Figs 11 and 7) Besides the dilatations observed in the main trunks such dilatations were rather often found in side branches, the proximal dilated part of the branch tapering into a pronounced constriction (Fig 8) Some of the visible arteries would show a more uniform constriction without dilatations, still others looked widely open,

often showing slight additional dilatations in several places. In many cases a very large percentage of the visible arteries showed the pattern of focal spasm alternating with dilated segments. When the observations were continued it was found that in some rats the constrictions and dilatations initially formed at the start of the angiotensin infusion would persist in the same location for several hours whereas in other rats the pattern would disappear in some or most of the initially affected vessels.

Injections of colloidal carbon (0.1 or 0.2 ml) were given through the femoral vein catheter at different times during the run of the infusion, often about 15 minutes after the start of the experiment. Some times 0.1 ml was given at later stages. The injection imparted a black colouring to the blood stream incidentally allowing the observation that none of the severely constricted parts were completely closed up since the carbon spread freely over the whole of the visible vasculature. After a short time the black colour became of decreasing intensity, thus allowing carbon deposits in the vascular walls to be recognized. The important finding, repeated in all experiments where carbon deposits were found, was that *deposits of carbon are never found in the spastic constricted parts of the arteries but predominantly in clearly dilated segments* (Figs 9, 10, 11, 12). A definite site of predilection was the proximal dilated part of the intestinal arteries but very often dilatations in the course of the artery over the intestinal surface showed carbon deposits. Some deposits were observed in apparently widely open arteries (without constricted segments) especially in slight dilatations on the otherwise even contour. The deposits were seen as streaks or dots in the wall of the artery just as described for the fixed and cleared preparations in part A of this paper. When the injection of carbon was repeated at later stages additional deposits were often formed either in already labelled segments or in not hitherto labelled dilatations.

In two of the experiments very few arteries showed spasm and dilatations. Only a single deposit was observed in a dilated segment.

In some experiments diffuse labelling indicating increased permeability of minute vessels was observed just as seen in the fixed preparations. At times these deposits were located in parts of the intestine where the arteries seemed relatively open without severe constriction.

The degree of arterial and diffuse labelling was quantitatively much varying in the different experiments. In many experiments a very large number of dilated segments were visible but by no means all of these became labelled.

#### 9. Infusion of Angiotensin (about 1.7 Micrograms per kg per min) into Intact Rats

The experiment was next repeated in four rats with intact kidneys. In three of these infusion of the same high dose of angiotensin imme-



12

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diately produced the same pattern of constrictions and dilatations as seen in the nephrectomized group, and injections of carbon gave rise to the formation of mural deposits exclusively in dilated segments

In two of these experiments diffuse labelling occurred, either close to dilated proximal segments of the arteries or related to arterial trunks without severe constrictions

The described phenomena are thus readily inducible in rats with intact kidneys

### 3 *Determination of Time Intervals Needed for the Formation of Mural Deposits of Carbon in the Intestinal Arteries*

In four experiments 0.1 ml of colloidal carbon was injected about one minute before the infusion of angiotensin (same dosage) was started. A stop watch was started when the blood pressure rise occurred, and the field of vision was closely searched, with this dose of carbon it is quite possible to recognize mural deposits even when carbon particles are circulating in the blood stream

In one rat with intact kidneys the first definite deposit was observed after 50 seconds (Fig. 11), the next after 90 seconds, after five minutes several deposits were visible in dilated parts. In another experiment the first deposits were found after 150, 180 and 210 seconds

Two experiments were performed in nephrectomized rats. The first three deposits were observed after 230, 275 and 330 seconds in one of these, the other rat showed very few deposits, the first one being recognized 420 seconds after the start of the angiotensin infusion

Hyperpermeability of dilated segments of the intestinal arteries can thus be recognized during the first few minutes after the induction of acute severe hypertension

### 4 *Infusion of Lower Doses of Angiotensin (0.1-0.3-0.6 Micrograms per kg per min) into Rats with Intact Kidneys*

Five experiments were performed. At the two lower dosages only moderate constrictions and dilatations were visible. A single carbon deposit was recognized in a moderately distended segment in one of these three experiments. At the highest dosage one rat showed the previously described constriction-dilatation pattern with subsequent carbon labelling of dilated parts, the other rat showed a rather uniform vasoconstriction and no deposits were observed. No clear relationship between dosage and pressure level was found

### 5 *Effects of Lowering the Blood Pressure during Infusion of Angiotensin into Nephrectomized Rats*

The previously described pattern of constrictions and dilatations was induced by infusion of angiotensin (about 1.7 micrograms per kg per min)

The effects of stopping the infusion was studied in three rats. The blood pressure decreased gradually and a gradual disappearance of the constrictions and dilatations was observed, after 5-10-15 minutes the pattern was no more recognizable. In two of these rats the infusion was then started again, the constrictions and dilatations reappeared in many of the arteries, apparently in the same localizations as before.

The effects of bleedings and retransfusions were studied in three rats, with the angiotensin infusion running continuously the arterial pressure was lowered by bleeding of the rat by way of an additional catheter placed in the femoral artery. The dilated segments showed a progressive diminution of diameter and the constricted parts showed a further narrowing, but the pattern of alternating spasm and dilatation could be recognized in some arteries at least for a time after reaching normal or subnormal pressures, tending finally to disappear leaving the arteries as severely constricted strings. With subsequent retransfusion the pattern could be reestablished in most of the arteries in two of the rats, in most places the constricted and dilated segments seemed to reappear at the same locations along the course of the arteries as before the bleeding procedure.

Even with this heavy dosage of angiotensin, a steep pressure fall occurs in spite of continued infusion if 0.2 mg of *hydralazine* (Aapresolin CIBA) is injected intravenously. The pattern of constrictions and dilatations was observed in two rats during the pressure fall (from 140-150 mm Hg down to 50-60 mm Hg in six to eight minutes) induced in this way, the pattern disappeared within about five minutes.

In order to study the permeability characteristics of the intestinal arteries in rats showing no elevation of the blood pressure in spite of continuous administration of angiotensin ten additional experiments were performed. In the first five of these acute hypertension with the associated constriction/dilatation pattern was induced by means of angiotensin in the usual dosage, and the angiotensin infusion was kept running during the whole of the experiment. After five

Nepresol (CIBA) in  
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100 mm Hg within 10 minutes. At this time an injection of colloidal carbon was given.

After the injection of Nepresol the severely spastic segments could be observed to open up and many arteries regained their normal contour, but in some arteries the dilatations formed at the start of the experiment were still visible after normal blood pressure values had been obtained. In one of the rats practically all arteries regained their normal appearance and no labelling took place. In two rats one or two carbon deposits were observed, localized to persistent dilatations. Finally, two rats showed a fair number of carbon deposits. Some of these were localized in typical dilatations, separated by intervening, more narrow

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After the injection of Nepresol the severely spastic segments could be observed to open up and many arteries regained their normal contour but in some arteries the dilatations formed at the start of the experiment were still visible after normal blood pressure values had been obtained. In one of the rats practically all arteries regained their

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segments, other deposits were found to occur in apparently widely open arteries, especially in very slight dilatations on these vessels. During the period in which the carbon particles were circulating, the blood pressure was in the range 70–115 mm Hg. It would thus seem, that the persistence of an abnormal pattern in the vasculature may allow a limited number of mural deposits to be formed even with the systemic arterial pressure inside the normal range.

In five experiments 0.35 mg of Nepresol was given 10 minutes before the start of the angiotensin infusion, the pressure was lowered to around 40–50 mm Hg. When the angiotensin infusion was started the pressure rose sharply to 140–150 mm Hg, but only for a few minutes, and the pattern of constrictions and dilatations disappeared quickly in four of the rats. The angiotensin infusion was kept running, but the pressure was kept in the range 60–125 mm Hg by the effect of dihydralazine. Colloidal carbon was given after 15 minutes of angiotensin infusion, but no deposits were found in the arteries observed. In one rat, however, although the blood pressure was well controlled (not exceeding 90 mm Hg), the constriction/dilatation-pattern did not disappear completely. Two carbon deposits were observed in an intestinal artery, lying in an open segment, the branches of which were constricted further distally.

#### 6 Administration of Rat Kidney Extract to Nephrectomized Rats

Rat kidney extract was administered subcutaneously to one, intravenously to another nephrectomized rat. In both moderate constrictions and dilatations were found to develop in some arteries, with subcutaneous administration these changes developed very slowly and were not pronounced until six hours after the injection of kidney extract, although the blood pressure had attained a level of 150 mm Hg after 1½ hours and 160–170 mm Hg after 3½ hours. After injection of carbon labelling occurred, again only in dilated segments.

#### 7 Administration of Noradrenaline to Nephrectomized Rats

Two experiments were performed using the same dosage as in the experiments reported in the preceding paper (about 3.4 micrograms per kg per min). The vasoconstriction was more uniform than with infusion of angiotensin, but a few moderately distended segments were observed in the proximal parts of the intestinal arteries. Labelling with carbon was observed only in such dilated segments.

Three experiments were performed with a dosage of 3.4 micrograms per kg per min, the blood pressure decreased rapidly after an initial pronounced rise. In one rat constrictions and dilatations were visible initially, but after a short time the arteries were rather uniformly constricted. Two rats showed a pronounced constriction/dilatation pattern in some of the visible arteries, much like that seen with angiotensin.

Carbon deposits were found in pronounced dilatations of the proximal parts of the arteries and in dilated segments further distally. also diffuse deposits in minute vessels were found

### III Infusion of Methoxamine into Nephrectomized Rats

As a further parallel to the studies reported in the preceding paper, methoxamine infusion (about 125 micrograms per kg per min) was performed in three nephrectomized rats. Varying types of reaction were observed in the intestinal arteries, which could show a uniform constriction or a pronounced dilatation in the proximal region with severe constriction in the more distal parts or the pattern of alternating constricted and dilated segments along the course of the artery.

Carbon deposits were predominantly formed in the proximal distended segments and occasionally in more distally located dilatations. No deposits were seen in the constricted segments. Diffuse deposits were found in two of the experiments as a sign of increased permeability in smaller vessels.

### 9 Control Experiments Infusion of Saline into Nephrectomized Rats

Two experiments were performed in nephrectomized rats receiving intravenous saline infusion in the same volume (about 0.4 ml per hour) as used in the angiotensin experiments. No spasm or pronounced dilatation was observed during four to five hours of observation, in one of the rats a single artery showed four minor dilatations in the beginning of the experiment, but they disappeared after a short time. Carbon injections after three or four hours were not followed by any formation of deposits.

## DISCUSSION

The phenomenon referred to as "vascular labelling" is a process

which indicates a local increase of permeability. The carbon particles are used as a colloidal tracer substance indicating the pathways followed by plasma escaping from the intravascular compartment and the "vascular labelling" phenomenon constitutes a parallel to the previously described occurrence of deposits of fluorochrome tagged serum proteins in arterial walls during repeated episodes of acute hypertension (Giese 1961).

The most important fact derived from the combined application of vital microscopy and the vascular labelling technique to the study of acute hypertension is the very consistent observation, that the deposition of carbon in the arterial wall takes place preferentially in dilated



segments of the small arteries, carbon deposits were never observed in severely constricted regions. The sites of predilection for the formation of mural deposits were the distended proximal segments of the intestinal arteries and pronounced dilatations further distally, at times deposits were observed in uniformly open arteries, often in additional small dilatations along the course of the vessel.

When trying to understand, why the increased permeability of the arterial walls is manifested only in dilated or at least widely open segments of the intestinal arteries, whereas segments showing constriction are never affected, some basic difficulties are encountered. In the first place, no precise explanation is available concerning the question why the arteries react in this peculiar way, showing constrictions in some places, dilatations in other places, when the vascular tone and the intraluminal pressure is increased by the administration of pressor agents. Secondly, although the systemic arterial pressure has been recorded, the intraluminal pressure in a given segment showing labeling is unknown in the absence of direct measurement. On the other hand it is quite clear, that the tangential tension in the wall of a dilated segment can be several times larger than the tension in the wall of an immediately adjacent constricted segment, since this tension is determined by the product of the intraluminal pressure and the radius (Wolf 1952).

The most reasonable assumption to be derived from the experimental findings would seem to be, that the essential factor is a distension of the structures of the arterial wall. The anatomical structures so influenced have not been identified in this study, but it would be reasonable to suggest changes in the intercellular endothelial junctions, the internal elastic lamina or/and the subendothelial basement membranes.

This concept, putting the induction of increased permeability of the arterial wall down to physical forces, would make the assumption (Giese 1962 and 1963), that the renin-angiotensin system might have a general permeability-increasing effect on vascular endothelium, rather untenable. Also, the experiments have shown, that focal increases of vascular permeability may be induced by other pressor agents.

When evaluating the experiments showing, that a limited number of carbon deposits may be formed in still dilated or at least widely open arteries during the infusion of angiotensin even at a time, when the blood pressure has been lowered by pharmacological means, it must be recognized that the haemodynamic situation created in a circulatory system under the joint influence of a pressor and a vasodilator agent is rather complex, and again direct measurement of the pressure locally in a given segment showing deposits would be necessary for a more complete analysis. But the decisive point seems to be, that an abnormal pattern is maintained in the vasculature by angiotensin even after lowering of the systemic arterial pressure.

Carbon deposits in the wall of small arteries have been recognized

within the first few minutes after the induction of acute hypertension with angiotensin. Thus astonishing rapidity in the development of hyperpermeable parts of the arterial system points strongly to the assumption that a deposition of plasma constituents in the vascular wall is a primary occurrence in the chain of events leading to acute hypertensive vascular damage. The very short time intervals needed for the development of mural deposits make it highly unlikely that the development of increased permeability should be regarded as a phenomenon dependent upon prior degeneration of the smooth muscle cells of the media. It seems much more likely that the focal hyperpermeability develops at once when the necessary physical conditions come into presence.

Besides the carbon deposits in the arterial walls, diffuse deposits of carbon indicating foci of increased permeability in smaller vessels have been observed during vital microscopy and in fixed and cleared preparations. This finding is in accordance with the frequent presence of mesenteric and intestinal oedema in rats exposed to angiotensin (and methoxamine). These diffuse deposits are very often located in close relation to dilated labelled arteries, especially at the mesenteric border and often such dilated arteries seem to lead directly to the diffuse deposits (Fig. 2). No precise definition of the type of minute vessels involved in the formation of these deposits has been obtained and the observations do not permit final conclusions concerning their pathogenesis. A reasonable hypothesis for further study would be that the increased permeability of these small vessels is due to an elevation of intraluminal pressure. Dilatation under high pressure of the artery supplying the area in question might allow the high intraarterial pressure to be transmitted distally thus allowing a pressure rise with dilatation and increased permeability of these small vessels. This concept would also fit well with the observations during vital microscopy.

The studies reported here are only concerned with observations during very acute hypertension. Therefore it is of the greatest interest that Byrom (1954, 1958a,b) has described the occurrence of a pattern of alternating severe constrictions and dilatations in the intestinal arteries of rats suffering from severe renal hypertension with or without hypertensive encephalopathy. His pictures of the intestinal vascular pattern are completely like the vital microscopic picture observed after induction of acute hypertension with angiotensin. The pattern of focal spasms and focal dilatations has further been observed in the cerebral arteries (Byrom 1954) during hypertensive encephalopathy and a similar pattern was observed in the retinal arteries of hypertensive rats by the same author (Byrom 1963).

Byrom's concept that the vascular lesions in human and experimental malignant hypertension are caused by repeated vascular crises involving a state of severe spasm seems very well supported by his elegant investigations. In all of the experimental situations studied by

segments of the small arteries, carbon deposits were never observed in severely constricted regions. The sites of predilection for the formation of mural deposits were the distended proximal segments of the intestinal arteries and pronounced dilatations further distally, at times deposits were observed in uniformly open arteries, often in additional small dilatations along the course of the vessel.

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Carbon deposits in the wall of small arteries have been recognized

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droxytryptophan in large amounts and of inducing the carcinoid syndrome (Sandler *et al* 1961), but the argentaffin reaction is nonetheless often negative in bronchial carcinoids Weiss & Ingram (1961), for example, observed a positive argentaffin reaction in but one of 5 bronchial carcinoids

Clearly, therefore, some carcinoids for obscure reasons exhibit a negative argentaffin reaction In most papers on negative argentaffin reactions it is not mentioned whether other histochemical procedures for demonstrating enterochromaffin were attempted Hence, in order to seek to elucidate the significance of negative argentaffin reactions in carcinoids, it was deemed worth while to compare the results of various histochemical reactions in a fairly large series of carcinoids The methods were checked by applying them to enterochromaffin cells in normal human appendix

## MATERIAL AND METHODS

The investigation was performed on a series of 27 carcinoids from the tumour register of the Pathological Laboratories (Gothenburg) The series included all the carcinoids diagnosed by operative biopsy over the years 1954 through 1963 after elimination of any cases in which the diagnosis was based on histological grounds (carcinoids found at autopsy were excluded since enterochromaffin substance is known to disappear after death) The material was available in the form of unfixed and formalin fixed sections 4  $\mu$  thick

For argentaffin reaction the sections were stained with 0.5% methenamine solution for 10 minutes and then incubated at 60°C for 1 hour

For chromaffin reaction the sections were stained with 0.5% chromaffin solution for 10 minutes and then incubated at 60°C for 1 hour

For argentaffin reaction after 140 minutes' incubation was also examined after 160 minutes' incubation when the connective tissue stroma of the tumours was examined

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University of Gothenburg Sweden

## ENTEROCHROMAFFIN REACTIONS IN INTESTINAL CARCINOID TUMOURS

By

LENNART ENFRBÄCK and YNGVE OLSSON

Received 21 iv 64

Since both enterochromaffin cells and carcinoid tumour cells after formalin fixation exhibit granules with a series of histochemical reactions in common, it has been postulated that carcinoids take origin from enterochromaffin cells. These reactions are known collectively as enterochromaffin reactions, and the substance they reveal has been termed enterochromaffin. The most important reactions include the argentaffin reaction, diazo reactions and ultraviolet fluorescence. A less specific reaction is Schmorl's ferricyanide method and it yields a positive outcome also with other substances capable of reducing ferricyanide to ferrocyanide, for example melanin and sulfhydryl groups (Pearse 1960). Strong evidence has been accumulated suggesting that the enterochromaffin substance is a condensation product of formalin and serotonin (5-hydroxytryptamine). Thus it has been demonstrated that carcinoid tumours produce and store serotonin (Lembek 1953, Pernow & Waldenström 1954), that reserpin administration is accompanied by parallel reductions of the serotonin quantity and enterochromaffin reactivity in the intestines of some animal species (Benditt & Wong 1957) and, in model experiments, that formalin treated serotonin exhibits positive enterochromaffin reactions (Benditt & Wong 1957, Holcenberg & Benditt 1961).

A variety of methods are accordingly available for demonstrating enterochromaffin substance in carcinoids, but the argentaffin reactions has become dominant. A positive argentaffin reaction has been deemed essential for the diagnosis of carcinoids; indeed, it has even been proposed that the term carcinoid should be replaced by "argentaffin carcinoma" (Willis 1960). The literature, on the other hand includes several reports of non argentaffin, intestinal and gastric carcinoids, even cases in which the tumour tissue was found to have a high serotonin content (Thorson 1958). Like intestinal carcinoids, bronchial carcinoids are capable of producing serotonin and its precursor 5 hy-

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The investigation was supported by grants from the Swedish Cancer Society. Diazo dyes were supplied by courtesy of Imperial Chemical Industries Ltd Dyestuffs Branch Sweden.

reaction. For unknown reasons a tumour with a positive fresh diazo reaction showed a negative reaction with Fast Garnet GBC. The strength of the reactions varied widely from one part to another of the sections but were usually most marked marginally in the tumour lobes. Owing to the few occasions when biochemical tests had been carried out on the patients, the strength of the reactions could not be correlated to any indications of serotonin production. Interesting, however, is that the most reactive tumour was a metastasis to the liver from a primary focus of unknown site.

TABLE 2  
*Histochemical Reactions of 27 Carcinoid Tumours*

| Site            | No. of tumours | No. of tumours with positive reactions |              |                    |                          |
|-----------------|----------------|--|--------------|--------------------|--------------------------|
|                 |                | Argentaffin                            | Ferricyanide | Fast red B (fresh) | Fast garnet GBC (static) |
| Appendix        | 18             | 14                                     | 16           | 18                 | 18                       |
| Small intestine | 5              | 3                                      | 5            | 5                  | 4                        |
| Metastases      | 4              | 4                                      | 4            | 4                  | 4                        |
| Total           | 27             | 21                                     | 27           | 27                 | 26                       |

The argentaffin tumours presented a brown to black, fine-grained silver precipitate in the cytoplasm of the tumour cells, no unspecific silver precipitate could be observed in the connective tissue stroma. After prolonged incubation, the non-argentaffin tumours exhibited unspecific silver deposits in the connective tissue stroma but the reaction in the tumour lobes remained negative.

The diazo methods imparted an orange to red tint to the granules in the cytoplasm of the tumour cells. Enterochromaffin cells were consistently coloured red. Even within the same tumour some areas contained orange cells and others red cells, the latter giving the impression of being more densely granulated.

Schnorr's ferricyanide method caused the tumour cells to stain deep blue.

The appearance of the various reactions in a typical case is illustrated in Figs 1, 2, 3, and 4.

## DISCUSSION

The present investigation on a series of 27 carcinoids demonstrates that out of 27

of 11 biopsy specimens, argentaffin, diazo and ferricyanide reactions in 7 out

th

and a condensation pro-



yellow colour which is not particularly disturbing. These observations are in agreement with those reported by Lillie, Greco-Henson & Cason (1960).

Schmorl's ferricyanide reaction was performed according to Pearce (1960).

The various methods were compared by testing them on human appendix. The argentaffin, fresh diazo and ferricyanide reactions were each applied on two of six consecutive serial 4  $\mu$  sections from each of five normal human appendices in the order given in Table 1. Typical enterochromaffin cells with basal granulation were observed in the deeper layers of the glandular epithelium in all sections. The total number of enterochromaffin cells in each section was counted and the results are given in Table 1. The diazo method tended to give a smaller number of cells than the argentaffin and ferricyanide method but the difference is not statistically significant.

When material from carcinoid tumours was tested a section of normal human appendix was placed in each cuvette and served as a control.

TABLE 1  
Comparison Between the Reactions on Normal Human Appendices

| Section No                                  | Consecutive 4 $\mu$ sections |                |                |                |                           |                |
|---|------------------------------|----------------|----------------|----------------|---------------------------|----------------|
|   | 1                            | 2              | 3              | 4              | 5                         | 6              |
| Entero-chromaffin cells,<br>Mean $\pm$ S.E. | 17.2 $\pm$ 4.2               | 17.3 $\pm$ 3.1 | 18.1 $\pm$ 3.8 | 14.1 $\pm$ 2.5 | 18.7 $\pm$ 4.1            | 13.5 $\pm$ 2.2 |
| Treatment                                   |                              |                |                |                |                           |                |
| Section No 1                                | Argentaffin reaction         |                |                | Section No 4   | Diazo reaction Fast Red B |                |
| Section No 2                                | Ferricyanide reaction        |                |                | Section No 5   | Ferricyanide reaction     |                |
| Section No 3                                | Argentaffin reaction         |                |                | Section No 6   | Diazo reaction Fast Red B |                |

## RESULTS

In this series of 27 carcinoid tumours, 18 were located in the appendix, 5 in the small bowel, and 4 were carcinoid metastases to liver lymph node and mesentery from primary tumours of unknown site. While 11 of the 18 appendix carcinoids extended to the serous coat, the rest invaded only the submucous and muscular coats. In one case tumour tissue had invaded the ovary. One of the appendix tumours exhibited multiple metastases to the mesentery. At the operation 2 of the small-bowel carcinoids were found to have metastasized and showed tumour growth in the serous coat. The remaining carcinoids of the small bowel were small and had been encountered accidentally.

Histologically all the tumours exhibited a typical carcinoid structure, with closely spaced clusters of monomorphic and cytoplasm-rich tumour cells separated by a sparse connective tissue stroma.

Urinary 5-HIAA estimation had been performed preoperatively in five cases, the 24 hour output being abnormally high (80.5 and 41 mg) in two patients with metastasizing carcinoids of the small bowel.

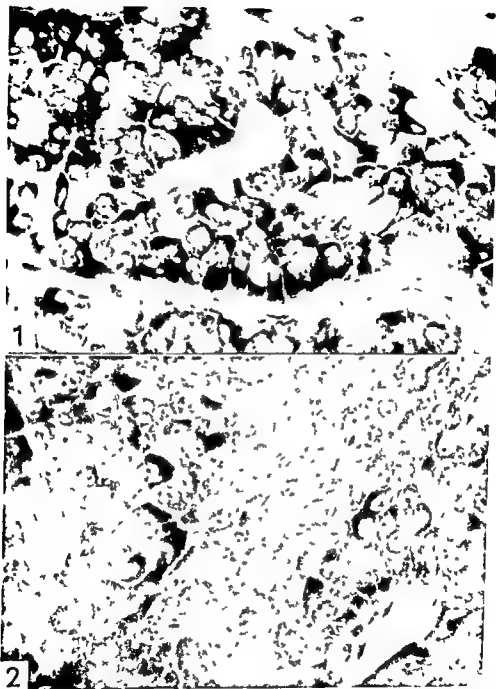
The results of the histochemical reactions are shown in Table 2. The fresh diazo reaction and the ferricyanide reaction were positive in all the tumours, while six of the tumours exhibited a negative argentaffin



Figs 3 &amp; 4

*Fig 3* Same tumour as in *Fig 1* Schmorl's ferricyanide reaction & nuclear stain (cytoplasmic granules stained blue owing to reduction of ferricyanide to ferrocyanide. Cell boundaries somewhat indistinct because of slight diffusion  $\times 518$

*Fig 4* Same tumour as in *Fig 1* Alkaline diazo reaction (Fast Garnet GBC) and Ehrlich's haematoxylin as nuclear stain. The majority of tumour cells in this field present powder fine orange coloured granulation but the darker cells in the centre of the figure are densely granulated and have a brick red colour  $\times 518$



Figs 1 2

- Fig 1* Carcinoid liver metastasis. Argentaffin reaction. Faintly stained nuclei (Safranin O). Black silver deposits in the tumour cells, especially those located along the margins of the tumour lobes. No unspecific silver deposits in the connective tissue stroma.  $\times 518$
- Fig 2* Same tumour as in Fig. 1. Alliline diazo reaction (Fast Garnet GBC). Weakly staining nuclei (Hehrich's haematoxylin). Orange to brick red cytoplasmic granulation.  $\times 518$

The negative argentaffin reactions, however, demand special consideration. Our series included no less than 6 out of 27 cases in which the argentaffin reaction was negative despite positive diazo and ferricyanide reactions. The precisely controlled length of the incubation period, to avoid the appearance in the sections of unspecific silver deposits, might conceivably have reduced the sensitivity of the silver impregnation method. Actually this could not have been the case for sections from all non-argentaffin tumours were reincubated until unspecific silver deposits appeared without the tumour cells themselves showing positive reactions. Furthermore, the comparison of the methods on appendix demonstrates that the argentaffin reaction is at least as sensitive as the other reactions for the purpose of visualizing enterochromaffin cells. The only plausible explanation left would seem to be that the argentaffin reaction on the one hand and the diazo and ferricyanide methods on the other have dissimilar chemical substrates, implying that they demonstrate a chemical difference between carcinoid tumour cells and enterochromaffin cells. Before any definite conclusions about this matter can be drawn, however, it will be necessary to study in greater detail the behaviour of these reactions in carcinoids, especially to compare directly the serotonin content of the tumours with the argentaffin and diazo reactions. Such investigations do not seem to have been performed, and it would probably be difficult to collect a reasonably large series in view of the fact that these studies would require the carcinoid to be diagnosed prior to extirpation of the tumour.

Thus, even if the discrepancy between the argentaffin reaction on the one hand and the diazo and ferricyanide reactions on the other lacks a satisfactory explanation at present, the results of the present investigation seem to indicate that the argentaffin reaction is *not* alone an adequate procedure for the histochemical determination of enterochromaffin material in carcinoids. The method should be combined with diazo reactions and the ferricyanide procedure. Besides, the argentaffin reaction is technically complicated and requires considerable experience. It can probably be dispensed with altogether for routine use while the diazo and ferricyanide procedures would seem to lend themselves well to incorporation into the ordinary programme of laboratory tests.

#### SUMMARY

In a series of formalin fixed biopsy specimens from 27 carcinoid tumours alkaline diazo reactions and Schmorl's ferricyanide reaction were positive in all cases, although the argentaffin reaction was negative in 6 out of the 27 cases. All reactions were compared with respect to their ability to visualize enterochromaffin cells in the —

duct of a 5-hydroxyindole and formalin. Studies on gelatin models by *Benditt & Wong* (1957) disclosed that the methods adopted here are capable of demonstrating the presence of serotonin in concentrations as low as 1 mg per ml. Negative histochemical reactions would in other words imply a serotonin content below this threshold of sensitivity. The strength of the reactions varied widely in our series, from cases in which practically all tumour cells contained positively reacting granules to cases in which only small areas of the tumours exhibited positive material. This phenomenon, combined with reports of negative *enterochromaffin* reactions in carcinoids, could mean that the serotonin content of carcinoids varies strongly. There are other reasons for assuming that this is so. In patients with carcinoid syndrome the manifestations believed to be associated with rises in the serotonin level of the blood occur paroxysmally and the urinary excretion of serotonin metabolites varies greatly from day to day, suggesting that the serotonin production in the tumours is characterized by alternating phases of storage and maximal secretion. Moreover, direct serotonin analysis in carcinoids have also yielded very variable values. Thus, *Davies* (1962) found a serotonin content varying from 1.2 to 1500  $\mu\text{g}$  per gramme of tumour tissue. The former value applied to a carcinoid metastasis to the liver and does not exceed the serotonin content of normal liver tissue.

In this connexion the possibility should be taken into consideration that the histological preparation procedure might release serotonin from the tumours. Serotonin might be present in the tumour cells in a readily soluble form, but the condensation product of serotonin and formalin has low solubility in water and the processing media employed in paraffin techniques. Accordingly dissolution could take place during formalin fixation. A recently advanced histochemical procedure for localization of monoamines on the cellular level with the aid of freeze drying and formalin-vapour fixation (*Falck* 1962) demonstrates, among other things, fluorescence in mast cells of the same type as in *enterochromaffin* cells. After fixation in an aqueous solution of formalin and routine paraffin embedding mast cells usually exhibit no such fluorescence, indicating that dissolution of reactive material can take place during the ordinary histological processing, at least from mast cells.

Clearly, therefore *enterochromaffin* material demonstrated by regular methods may vary owing to technical as well as biological factors. Consequently negative histochemical reactions cannot rule out a diagnosis of carcinoid tumour in cases in which the morphological picture is not typical. Conversely, the results of the present investigation show that the serotonin quantity in carcinoids in practice rarely lies below the sensitivity thresholds of the *in vivo* methods and *Schmorl's* ferricyanide procedure after routine formalin fixation. Hence these methods are evidently of great value in the diagnosis of carcinoid tumours.

The Swiss Tropical Institute (Head Professor H Geigy), the Outward Clinic of the University of Basle (Head Professor O Gsell) and the University Department of Pathology General Hospital Malmö (Head Professor I Linell)

## MAST CELLS AND CORONARY HEART DISEASE<sup>1</sup>

*Relationship between Number of Mast Cells in the Myocardium,  
Severity of Coronary Atherosclerosis  
and Myocardial Infarction in an Autopsy Series of 672 Cases*

By

MICHEL FRAXEX and NILS H STERNBY

Received 23 iv 64

As many clinical and experimental studies have shown a possible relationship between mast cells and atherosclerosis we thought it worth while to study the relation between coronary atherosclerosis respectively myocardial infarction and the number of mast cells of the myocardium in a large autopsy series from a well defined population

Mast cells are important elements of the connective tissue. They vary in shape they have a round central nucleus and their cytoplasm contains basophil granules

Mast cells are said to be the chemical laboratory of connective tissue and to play an important rôle in the turn over growth and repair of this tissue

Atherosclerosis is a degenerative disease of the artery wall associated with changes in lipid metabolism and, perhaps in the blood clotting system. A common

this complex clearing factor or lipoprotein lipase but rather to a deficiency in the releasing mechanism of this enzyme since longterm parenteral treatment with heparin of hyperlipaemic patients does not produce any signs of exhaustion of the

<sup>1</sup> This work was supported by a grant of The World Health Organization section for Cardiovascular Diseases and by The Swiss National Fund for Scientific Research. The vessels were collected in a study supported by The Swedish National Association against Heart and Chest Diseases

it appeared that the use of elevated temperature during incubation in the silver solution necessitated precise control of the duration of the incubation period to avoid unspecific silver deposition. Carcinoids showing a negative argentaffin reaction were reincubated until an unspecific silver deposit appeared in the section but the tumour cells still remained negative. In human appendix, on the other hand, the argentaffin reaction visualized at least as many enterochromaffin cells as the other methods. The reason for these discrepancies in the argentaffin reaction between enterochromaffin cells and carcinoid tumour cells is obscure, but their mere existence implies that the argentaffin reaction alone is of limited value in the diagnosis of carcinoids.

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The physiology of mast cells has been studied by L. J. Gold (15 42 48) (33 36) who found that the number of mast cells is



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## MAST CELLS AND CORONARY HEART DISEASE<sup>1</sup>

*Relationship between Number of Mast Cells in the Myocardium,  
Severity of Coronary Atherosclerosis  
and Myocardial Infarction in an Autopsy Series of 672 Cases*

By

MICHEL FERNEX and NILS H STRANDBY

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As many clinical and experimental studies have shown a possible relationship between mast cells and atherosclerosis we thought it worth while to study the relation between coronary atherosclerosis respectively myocardial infarction and the number of mast cells of the myocardium in a large autopsy series from a well defined population

Mast cells are important elements of the connective tissue. They vary in shape they have a round central nucleus and their cytoplasm contains basophil granules

\* Wilander  
concentra  
apart from

Blood basophils are the only cells known to produce this mucopolysaccharide (3-9). The function of the mast cells however is complex. They produce also histamine (37-37-61) and their cytoplasm shows a high enzymatic activity (4-14-16-44-60)

As hypoparathyroidism does not appear to be the consequence of a lack of this complex clearing factor or lipoprotein lipase but rather to a deficiency in the releasing mechanism of this enzyme since longterm parenteral treatment with heparin of hyperlipaemic patients does not produce any signs of exhaustion of the lipoprotein lipase. Endogenous heparin might be the deficient releasing agent (19-64).

As mast cells produce endogenous heparin many authors have discussed

This might however, be the way to show the role played by the heparinocytes in the development of atherosclerotic diseases. A similar approach to this problem is counting of tissue mast cells.

*Curns & Constantinides* noted that experimental atherosclerosis was easy to induce in animals with few mast cells such as rabbits but not in rats which have a large number of tissue mast cells. They also studied these cells in sections (7  $\mu$ ) of the myocardium of man 60-90 years of age. They found fewer mast cells in cases with than in those without advanced atherosclerotic changes in the coronaries (10 11 12). *Wills Strickland & Paterson* later showed that the mast cell number in man remained unchanged until 24 hours after death (43). They noticed that the mast cell number was lower in cases with than in those without stenosis or thrombosis of the coronary arteries (50).

In some other studies the mast cells within the walls of more or less atherosclerotic arteries have been counted and seemed to be more numerous in diseased than in normal vessels. This localized mastocytosis might however be related to the local inflammatory process in the vessels where the number of lymphocytes is also increased (51 52).

The number of mast cell decreases at one and the same rate with age in human skin myocardium (33) and the adventitia of veins (68). On the other hand atherosclerosis increases with age. In African people where serum cholesterol values are low and coronary heart disease practically nonexistent the mast cells in the skin and myocardium are approximately twice as abundant as in Europeans where the incidence of myocardial infarction is high (21 23). It has also been shown that hypocholesterolaemia is common in generalized urticaria pigmentosa, a diffuse mast cell proliferation (24 54 62).

*Lempert Stein & Doyle* found the number of mast cells in the myocardium to differ from one part of the muscle to the other and therefore concluded that the counting of mast cells in a single or a few specimens was not reliable (39a). On the other hand in a larger series *Hellstrom & Holmgren* found a good correlation between different parts of the myocardium and also in different pieces of skin (33).

Many authors have stressed the role of mast cells in experimental atherosclerosis (11 13 27, 28 70). *Watson* could not induce atherosclerosis by the use of a high fat diet in rats treated with histamine liberators (71) substances which cause mast cells to disrupt (20 49 57 59). These mast cells are not destroyed but difficult to recognize on histologic slides (30). Also *Douglas et al* failed to induce experimental atherosclerosis by this method (13). Atherosclerosis could however be produced in the rat by combining a fatty diet and thiouracil which decreases the mast cell number and reduces their activity (25).

*P. Lerner* used worm infections and histamine liberators to stimulate mast cells in rats and noted an acceleration of catabolism of alimentary fats in rats with previously stimulated mast cells (26).

## MATERIAL AND METHODS

The material was collected from 794 consecutive autopsies from cases aged 18 to 98 years performed at the General Hospital Malmö, Sweden. The hospital serves a city with about a quarter of a million inhabitants. About 60 per cent of all deaths within the city occur in the hospital and the autopsy rate is 99 per cent. Most of the autopsy cases were above 65 years of age and no legal cases were included. The autopsies were performed 2-48 hours after death, mostly within the first 12-18 hours.

The coronary arteries were opened longitudinally by the prosector and then dissected from the heart by technicians. The vessels were cleaned from fat, fixed in 10 per cent neutral formalin for 12-24 hours and placed in transparent plastic bags with a small amount of formalin according to a method described by *Holman et al* (34). With the specimens preserved in this way evaluation could be postponed until all the specimens had been collected.

The main trunk of each of the coronary arteries was graded separately according to the following scale:

- Grade 0 no lesions or a few lipid streaks
- Grade 1 numerous lipid streaks with or without a few fibrous plaques
- Grade 2 numerous fibrous plaques with or without very small calcifications but without any complicated lesions i.e. bleedings, ulcerations and thrombi

Grade 3 small to fairly extensive calcifications with or without small complicated lesions

Grade 4 extensive calcified and/or complicated lesions

For each case the sum of the three grading figures was calculated, and the cases were then divided into three types of coronary atherosclerosis according to the following scale

Grading sum 0-3 slight coronary atherosclerosis

Grading sum 4-6 moderate coronary atherosclerosis

Grading sum 7-12 severe coronary atherosclerosis

For mast cell counting in the myocardium specimens were taken at the base of the front and the lateral wall of the left ventricle one from each of the two pieces

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toluidine blue stain which appeared dark violet on a very light blue field were counted in 40 adjacent fields measuring  $1/4 \text{ mm}^2$  each in both pieces and the average value was reported per square millimeter myocardium

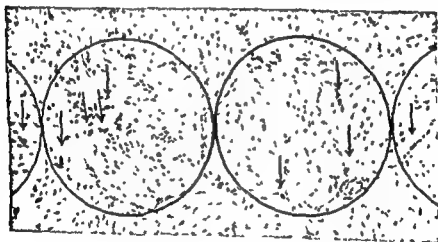


Fig 1

The appearance of mast cells in the myocardium (Toluidine Blue 75 X)

192 cases were excluded for various reasons

in 64 cases pieces of myocardium were missing or consisted of only necrotic or granular connective tissue

in 29 cases macroscopic description of the myocardium or other important pathological data were missing

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## RESULTS

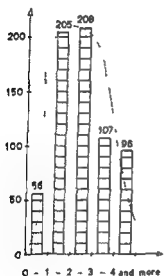
*Number of Mast Cells in the myocardium*

The number of mast cells ranged between 0.1 and 11.5 per  $\text{mm}^2$  of myocardium. The number of cases in different age groups and in 5 groups according to the mast cell number is given in Table 1.

TABLE 1  
*Distribution of 672 Cases According to Age and to Mast Cell Number in the Myocardium*

| Mast cells $\text{mm}^2$ | 0.1    |     | 1-3    |     | 2-3    |     | 3-4    |     | >4     |     | Total number of cases |
|--------------------------|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|-----------------------|
| Age groups               | Number | %   | Number | %   | Number | %   | Number | %   | Number | %   |                       |
| 18-60                    | 12     | 21  | 32     | 16  | 40     | 19  | 17     | 16  | 33     | 34  | 134                   |
| 61-70                    | 17     | 30  | 49     | 23  | 47     | 23  | 31     | 29  | 22     | 23  | 165                   |
| 71-80                    | 21     | 38  | 74     | 36  | 67     | 32  | 37     | 34  | 28     | 29  | 217                   |
| >80                      | 6      | 11  | 51     | 25  | 54     | 26  | 22     | 21  | 13     | 14  | 146                   |
| All ages                 | 56     | 100 | 205    | 100 | 208    | 100 | 107    | 100 | 96     | 100 | 672                   |

NUMBER OF CASES

NUMBER OF MAST CELLS/ $\text{mm}^2$ 

PERCENTAGE OF THE CASES

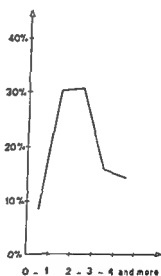
NUMBER OF MAST CELLS/ $\text{mm}^2$ 

Fig. 2

Distribution of 672 cases according to their mast cell number. To the left: absolute number of cases; to the right: percentual distribution.

In 61 per cent of the cases there was between 1 and 3 MC/ $\text{mm}^2$ . In about 8 per cent, less than 1 MC/ $\text{mm}^2$  was found. Fig. 2 shows the distribution of all the cases according to mast cell number. The cases with less than 1 MC/ $\text{mm}^2$  are said to have "few mast cells", the cases with

1-3 MC/mm<sup>2</sup> are said to have a normal mast cell count and the cases with more than 3 MC/mm<sup>2</sup> are said to have a large number of mast cells in their myocardium

### Mast Cell Number in Different Age Groups

In all age groups cases with a large or small mast cell number were encountered (Table 1). However the youngest age group which represents only 20 per cent of the cases includes 35 per cent of the cases with more than 3 MC/mm<sup>2</sup>. In the older age groups the proportion of cases with a high mast cell count is decreasing (Fig 3)

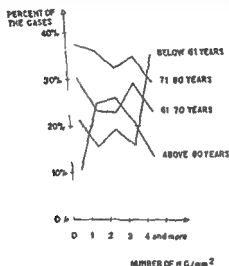


Fig 3

Percentual distribution of the cases in different age groups according to the mast cell number

### Mast Cell Number According to Sex

In this autopsy series the sex ratio was nearly 1/1 (49 per cent women). No sex difference in mast cell number was found (Fig 4).

There were only 28 women below 50 years of age of whom 12 can be considered as premenopausal (neither castrated nor receiving massive sex hormone therapy). The average mast cell number in these 12 cases was 2.86  $\mu$  r mm.

### Mast Cells and Severity of Coronary Atherosclerosis

Atherosclerotic changes in the coronary arteries were graded as slight, moderate or severe. The percentage of cases with slight coronary atherosclerosis decreased with age: 36 per cent of the cases less than 61 years

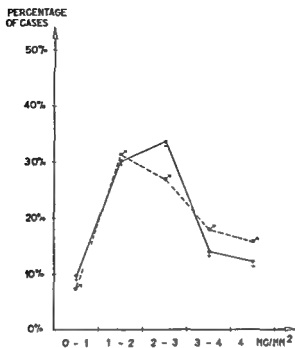


Fig 5

Distribution of mast cell numbers among men and women

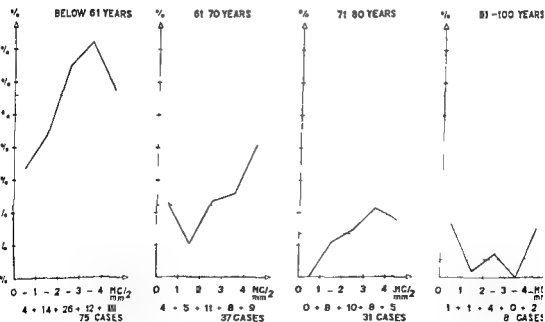


Fig 5

Distribution of cases with slight coronary atherosclerosis according to age and mast cell number

age 22 per cent of those between 61 and 70, 14 per cent between 71 and 80 and only 6 per cent more than 80 years of age. However, the percentages were different in most age groups when the number of mast cells was considered. Fig 5 shows that a high percentage of cases with light coronary atherosclerosis occurs not only in younger age groups, but also in subjects with a high mast cell count. This holds for ages up to 80 but in the oldest age group, only very few coronaries remain free from severe atherosclerotic changes.

Fig 6 shows the percentual distribution of the cases with slight, moderate and severe coronary atherosclerosis in relation to mast cell number. It is obvious that the cases with slight atherosclerotic changes often have a high mast cell count and conversely, that the cases with severe atheromatosis often have a low mast cell count.

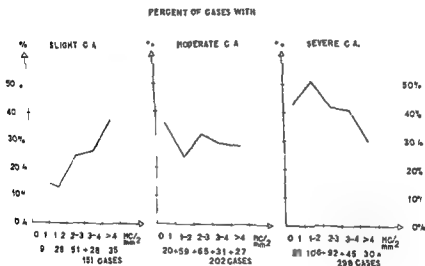


Fig 6

The relation between the severity of coronary atherosclerosis and mast cell number

#### *Incidence of Myocardial Infarction According to Age, Sex and Mast Cell Number*

Myocardial infarction both recent and old was recorded in 35.6 per cent of the 672 autopsies. It occurred most frequently between 61 and 70 years of age where the incidence was 45 per cent.

Of 151 cases with slight coronary atherosclerosis 11 per cent showed myocardial infarctions. In 202 cases with moderate coronary atherosclerosis the incidence was 23.5 per cent and in 298 cases with severe atherosclerosis it was 56.7 per cent.

The cases were equally distributed when related to the number of mast cells in the left ventricle 46 per



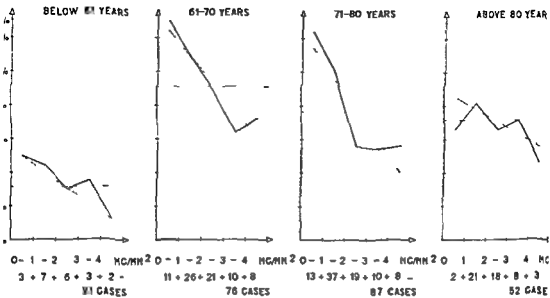


Fig 7

Incidence of myocardial infarctions in different age groups according to mast cell number. The dotted line represents the regression line.

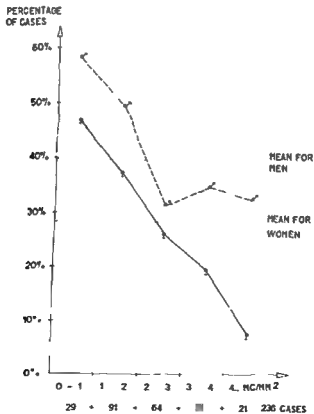


Fig 8

Incidence of myocardial infarction according to mast cell number in men and women.

cent of the cases with a low mast cell count ( $< 2 \text{ MC/mm}^2$ ), showed infarction, against 30 per cent of the cases with a "normal" mast cell count ( $2-3 \text{ MC/mm}^2$ ) and only 24 per cent of the cases with a high mast cell count ( $> 3 \text{ MC/mm}^2$ ). So the incidence of myocardial infarction is distinctly higher in cases with a low than in cases with a high mast cell number. This holds for all age groups (Fig. 7). The calculation of the regression line<sup>1</sup> for the different age groups showed that the difference in the incidence of myocardial infarction in individuals with more or fewer mast cells is highly significant for the age groups between 61 and 80, and nearly significant for the youngest group. It is not significant for the oldest age group.

The incidence of myocardial infarction was higher in men (39.8 per cent) than in women (28.3 per cent) in this series. In both sexes the incidence of myocardial infarction decreases with increasing number of mast cells (Fig. 8).

Of 672 cases 96 had more than  $4 \text{ MC/mm}^2$ . Myocardial infarctions were recorded in 21 (21.8 per cent) of these cases, 3 females and 18 males. In men with a high mast cell count, the incidence of myocardial infarction thus remains fairly high. Among the men was one case of essential hypercholesterinaemia and one of uric diathesis. These two diseases are inborn errors of metabolism frequently associated with coronary occlusion but the pathogenesis of coronary occlusion in these cases might be different from that in ordinary atherosclerotic disease. In 3 cases the cause of death was syphilitic aortitis, where the ischaemic heart disease was not due to coronary atherosclerosis. Eight died from different malignant diseases.

## DISCUSSION

The number of mast cells in the myocardium from the left ventricular wall was found to vary between 0.1 and  $11.5 \text{ per mm}^2$ . These values are about the same as those found in other white populations, e.g. from Canada (10) and Paris (21), but much lower than those in the Senegalese population of Dakar (21). For an "European Standard", one might consider  $0-1 \text{ MC/mm}^2$  as a low mast cell count,  $1-3 \text{ MC/mm}^2$  as a normal, and above  $3 \text{ MC/mm}^2$  as a high mast cell value.

For African populations, where the average value is  $5.2 \text{ MC/mm}^2$ , a high mast cell count starts at  $6 \text{ MC/mm}^2$ .

In our material no cases below 18 years of age were included. No significant difference in myocardial mast cell number appeared in the

In mice, however, an increase in the mast cell number with age has been reported (67).

<sup>1</sup> We are grateful to Prof. F. Linder, Geneva, for the statistical analysis (40).

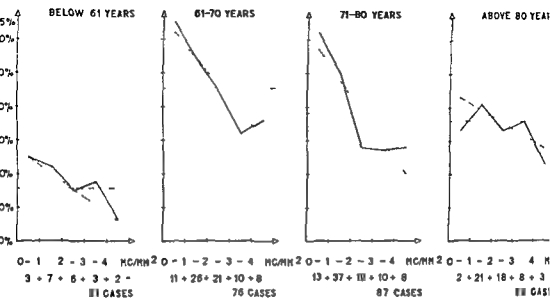


Fig 7

Incidence of myocardial infarctions in different age groups according to mast cell number. The dotted line represents the regression line.

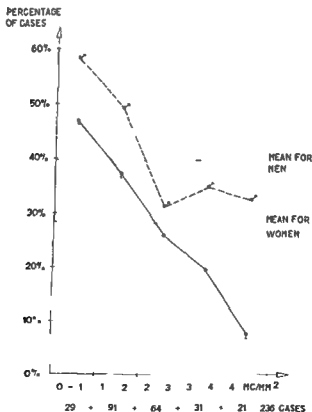


Fig 8

Incidence of myocardial infarction according to mast cell number in men and women.

The reduced incidence of myocardial infarction in subjects with many mast cells in the myocardium might be related to the activities of these cells. If so, it might be possible to reveal some "predisposing factor for atherosclerosis by measuring the excretion of histamine and or heparin metabolites in urine, the variation in mast cell specific enzymatic activity in blood or the mast cell number in a standard skin biopsy, since the number of mast cells in myocardium and skin vary in the same way (33).

## SUMMARY

In a large autopsy series from Sweden the relationship between coronary atherosclerosis, myocardial infarction and mast cell number of the myocardium was studied. The number of mast cells was found to be the same as in other white populations.

Cases below 80 years of age with a high mast cell number showed less coronary atherosclerosis than cases with a low number of mast cells.

The incidence of myocardial infarction recent and old in the age group 61-80 years (392 cases) was significantly lower for cases with a high mast cell count than for cases with few mast cells.

The relationship between the function of the mast cells and atherosclerosis is discussed.

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different age groups, although individuals with high mast cell count were more frequently found among younger people. In previous investigations sex differences in the mast cell number have been noted in individuals below 40 years of age (33). This age group is very small in our material. The twelve premenopausal women have about the same number of mast cells as the rest of the population.

A relationship was found between grade of coronary atherosclerosis and mast cell number in people less than 81 years of age. Subjects with higher mast cell count appeared to show less coronary atherosclerosis.

A statistically highly significant relation was also found between number of mast cells and incidence of myocardial infarction in the 392 subjects aged 61-80 years. Infarction occurred more frequently in cases with a low mast cell count. A similar result, but not statistically significant, was found in 134 cases below 60 years. In the 146 cases above 80 years a similar tendency was still demonstrated. 23 cases with myocardial infarction had more than 4 MC/mm<sup>2</sup>. This group included one case of essential hypercholesterolaemia with xanthomatosis, one case of uric diathesis and 11 cases of syphilitic aortitis in which the pathogenesis of coronary occlusion differs from that of ordinary coronary heart disease. In hypercholesterolaemia with xanthomatosis, the extremely high mast cell number might be interpreted as a reactive proliferation of mast cells to this dyslipaemia. The mast cells do not, however, succeed in influencing the lipid metabolism because heparin fails to reduce the hypercholesterolaemia (63).

Many authors accept that the disturbance in the lipid metabolism in coronary heart disease might be partly related to a reduced heparin production (19, 46, 66). A hypoplasia of the mast cell system could be the cause of a hypoheparinaemia.

Does the change in number of mast cells precede the atherosclerosis of the coronary arteries or is it the consequence of ischaemia or any other disturbance? Observations on the degree of coronary atherosclerosis in Africans, where myocardial infarction and angina pectoris is practically non-existent, the low serum cholesterol level in patients with diffuse idiopathic mastocytosis (24) and the parallelism of the decrease of the mast cell number in human skin and heart with ageing (33) argues for the first hypothesis, as do observations in laboratory animals where the reduction of the mast cell activity and number precedes the vessel changes (13, 25, 64).

Mast cells thus appear to be involved in the mechanism of defence against atherosclerotic degeneration of arteries. They interfere with coronary heart disease in two ways. In producing heparin mast cells accelerate the catabolism of the alimentary lipids and consequently increase the fibrinolytic activity and reduce the clotting tendency of blood (39, 45). The production of very active enzymes and histamine is another important function. Histamine released during ischaemia might prevent extensive necrosis by inducing local vasodilatation and development of collaterals.

The reduced incidence of myocardial infarction in subjects with many mast cells in the myocardium might be related to the activities of these cells. If so, it might be possible to reveal some "predisposing" factor for atherosclerosis by measuring the excretion of histamine and/or heparin metabolites in urine, the variation in mast cell "specific" enzymatic activity in blood or, the mast cell number in a standard skin biopsy, since the number of mast cells in myocardium and skin vary in the same way (33).

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## SARCOMAS IN ALBINO MICE INOCULATED WITH ROUS CHICKEN TUMOUR MATERIAL

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Rous sarcoma has been transplanted to various mammals, including mice. *Roskin* (1927) reported a fairly long temporary growth of such transplants in mice with blockaded reticulo endothelial system. Similar results have been obtained by *Greene* (1931) and *Ageenko* (1937), who transplanted the tumour to the brain of the mouse and by *Algire et al* (1938), who used diffusion chambers. In this laboratory subcutaneous transplants of the Rous chicken sarcoma (Mill Hill strain) to new born mice survived for a short time and showed signs of active growth during the first week (*Ahlström & Jonsson* 1962 a). Cortisone treatment did not affect the duration of survival of the transplants.

Previous experiments have thus produced no evidence of pathogenicity of the Rous sarcoma virus for mice and heterotransplants of the chicken sarcoma regressed after temporary growth. Besides this, most investigators have transplanted the tumour to immunologically shielded sites or used animals with impaired immunological reactivity.

In recent years however, several reports on the pathogenicity and tumour producing effect of Rous sarcoma virus in mammals have appeared (*Zilber & Kryukova* 1957, *Svet Voldavsky* 1957 and 1958, *Schmidt Rupp* 1959, *Svoboda & Grozdanovic* 1959, *Svoboda* 1961). *Schmidt Rupp* (1959) reported the development of sarcomas in heterozygous albino mice following intramuscular injection of Rous sarcoma material. The tumours could be carried in series in mice. The transplantable mouse tumour of *Schmidt Rupp* had a chromosome pattern different from that of the chicken sarcoma and similar to that usually found in mouse tumours (*Ising Iversen* 1960). *Ising Iversen* considered that "heterologous injection of Rous virus might have given rise to a virus induced tumour differing in chromosome pattern and histologic picture from the Rous sarcoma".

This paper deals with the results of an extended investigation on mice inoculated with the same strain of Rous sarcoma virus as that used by *Ising Iversen*. In a large series of investigation this virus strain has been shown to produce tumours in rats hamsters guinea pigs and rabbits (*Ahlström et al* 1962 1963).

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## MATERIAL AND METHODS

The Rous sarcoma material was originally obtained from Dr H Schmidt Rupp in Frankfurt on the Main. It has been maintained in this laboratory by serial passages in 2 to 3 week old White Leghorn chickens or in some periods kept at 65°C. Chicken sarcoma tissue was finely minced with scissors and suspended in 4 parts of Hank's solution (w/v) containing 100 units of penicillin and 0.1 mg of streptomycin per ml. After intramuscular injection of 0.5 ml of the suspension into the pectoral muscle of the fowl a tumour developed in a few days with the usual appearance of a Rous sarcoma. The tumour was transferred at about 14 day intervals. A suspension of chicken sarcoma tissue prepared in the same way was used for injection into mice.

Extracts of chicken sarcoma were prepared in the following way. Tumour tissue was thoroughly ground with sterile glass powder in a mortar or in a glass homogenizer and suspended in 4 parts of Hank's solution containing antibiotics as described above. After clarification in an MSF Super Maltex centrifuge for 15 minutes at 4000 r.p.m. the supernatant was centrifuged for 20 minutes at 10000  $\times$  g at 3°C in an International Colli Centrifuge. The supernatant was cautiously sucked off. This procedure was repeated twice. The final supernatant was used for inoculation of mice.

For the preparation of Seitz filtrates the usual sarcoma suspension or homogenate was centrifuged in the MSF centrifuge at 4000 r.p.m. after which the supernatant was passed through a Seitz filter.

The white mice belonged to a closed colony of Swiss mice which has been bred at the institute for many years. They were fed standard commercial pellets with an addition of milk and fresh greens.

## RESULTS

## 1 Inoculation of New born Mice with Minced Chicken Sarcoma

A suspension of finely minced chicken sarcoma was injected into 21 litters of mice, 0 to 4 days of age. Animals below 24 hours of age at the time of inoculation received 0.03 or 0.05 ml, the remainder 0.05 or 0.1 ml. The dose was given subcutaneously on the back, the needle being introduced via the left hind leg to reduce back flow. Nearly half of the animals died or were lost by cannibalism as a rule within the first week of the injection.

**Gross findings.** During the first week a swelling developed on the back of some of the animals similar to that seen after injection of a suspension of Rous sarcoma of the Mill Hill strain (Ahlstrom & Jonsen 1962a).

Of the surviving 72 animals however 23 developed progressively growing sarcomas at the site of inoculation. The first tumours appeared 19 days after the inoculation while one tumour could not be palpated until nearly 9 months after inoculation. Twelve of the tumours could initially be felt between the 19th and the 45th day.

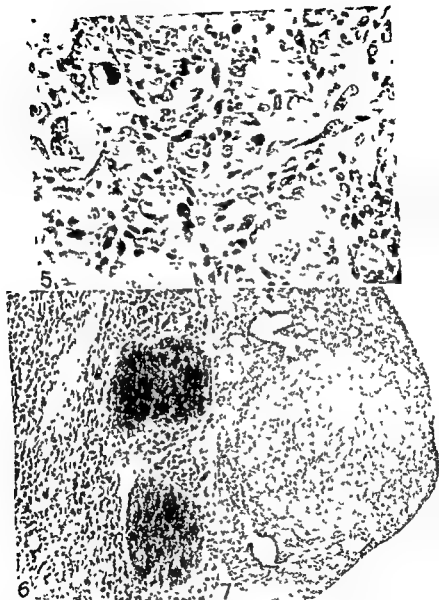
The tumours were circumscribed and firm initially non adherent

## Figs 1-5

Fig 1 Sarcoma on the back of a 19 day old mouse inoculated at the age of 2 days

Fig 2 Enlarged cells. Htx eosin  $\times$  160  
Fig 3 Higher magnification. Two types of cells are seen. Htx eosin

Fig 4 Network of argyrophil fibrils in the sarcoma. Papanicolaou  $\times$  400



Figs 5-7

Fig 5 Another part of the same tumour. Large cells with abundant cytoplasm among the elongated cells. Htx eosin  $\times 400$

Fig 6 Large distended vessels in the tumour tissue. Htx eosin  $\times 160$

Fig 7 Lung metastasis in a mouse. Htx eosin  $\times 160$

but later adherent to the musculature and the spine. They grew rapidly and within a few weeks they often involved the back and flanks of the host. The skin was often adherent to the surface of the tumour, but necrosis and ulceration were rare. In some animals small lumps, apparently along the needle track, could be felt caudal to the main tumour.

The animals gradually became severely disabled by the tumours and had to be killed. Necropsy showed the tumours to have infiltrated the spine, the bony pelvis or femur, the musculature of the back and the flanks as well as the thoracic wall with destruction of the latter and sometimes with involvement of the lungs (Fig 1). In some animals the kidneys and adrenals were surrounded by tumour masses bulging into the abdominal cavity, no ascites was, however, seen. The surface of the tumour was often smooth, polycyclic, and adjacent the subcutaneous tissue was oedematous and in some cases showed streaky confluent haemorrhages.

The tumours were relatively firm in the peripheral parts, while in the central parts they were soft. The cut surface was grey, moistened and lustrous. The larger tumours sometimes showed irregular areas of necrosis filled with blood.

Four mice showed small to peppercorn-sized firm, white tumour nodules on the surface of the visceral pleurae and in the lung parenchyma. The retroperitoneal lymph nodes were often swollen but apparently not invaded by the tumour. One mouse showed a peppercorn-sized tumour situated at the renal hilus and encroaching upon the ureter. No metastases were seen in other organs.

No cysts of the type described previously in rats and rabbits were seen.

*Microscopical findings* The tumours largely resembled spindle-cell sarcomas and were built up of three types of cells. The picture was dominated by crowded, spindle-shaped cells fairly rich in eosinophilic cytoplasm with rather large, oval nuclei with a vesicular chromatin structure and one or more large nucleoli. Less numerous, intermingled smaller cells with indistinct cytoplasm and small round or oval nuclei fairly rich in chromatin were also seen (Figs 2-3). Graceful connective tissue fibrils surrounding small groups of tumour cells were observed (Fig 4) in sections stained with van Gieson and in sections impregnated with silver according to Laidlaw. The softer parts of the tumours also contained numerous giant cells with voluminous cytoplasm, often with an eosinophil, homogeneous central portion and a more basophilic, vacuolated or streaky periphery. The cells showed one or more peripheral nuclei with a vesicular structure and large nucleoli (Fig 5). No cross striation could be observed. Staining for fat proved negative. The examination often gave the impression of a gradual transition between different types of cells. Centrally the larger tumours showed oedematous or reticular loosening and large or small irregular necrotic foci. No cysts could be observed, but tissues around and in the tumour some-

and grew progressively invading the thoracic wall and abdominal musculature. Histologically it was identical with the tumours described above. No gross or microscopic metastases were found. In the other animal the tumour appeared 6 months after inoculation, it grew slowly and had the histological character of a highly differentiated fibrosarcoma.

In addition a number of animals were inoculated with filtrates from the chicken sarcoma prepared in different ways. Two litters of eleven 2 day old mice were inoculated with filtrate prepared by centrifugation of a suspension of chicken tumour in an MSI centrifuge twice for 10 minutes at 4 000 r.p.m. after which the supernatant was filtered through a membrane filter Art 20 (Göttingen) (pore size  $1-3 \mu$ ). Eight months later a hazel nut sized tumour with the histological characteristics of an adenocarcinoma developed in the right axilla of one of the animals. It had probably originated from the sweat glands or mammary. Attempts to transfer the tumour to chicken proved negative.

A litter of six 1 day old mice were inoculated daily with 0.03 ml of the same type of filtrate on 4 consecutive days and then with 0.1 ml on 2 days. No tumour developed (observation period 13 months).

Four litters of altogether 29 animals aged 12 hours to 4 days were inoculated with filtrate prepared in the above mentioned way with chicken tumour as starting material. This tumour had developed following injection of sarcoma suspension from a mouse inoculated with chicken tumour. Nineteen animals survived, in 3 of them tumours appeared in the thigh and in the back within 8-13 months and gradually assumed the size of walnuts and metastasized to the lungs and pleura. All the tumours had the histological character of an adenocarcinoma of the same type as that just mentioned. Inoculation of mice and chickens with these tumours gave negative results.

Two litters of 12 animals (12 hours respectively 4 days old) were inoculated with filtrate prepared in the following way. The supernatant of chicken fibroblast cultures inoculated with high titred virus pool (titre  $> 10 000$  m.i.d./ml) and showing widespread foci were centrifuged twice at 3 000 r.p.m. in an MSI centrifuge after which the supernatant was filtered through Cell-filter grob (Göttingen) (pore size  $0.6-1.4 \mu$ ). All these animals survived and an adenocarcinoma of the type described above occurred in one of them.

Altogether 93 mice aged 12 hours to 7 days were inoculated subcutaneously with Seitz filtrate of a suspension of chicken tumours. In some cases the tissue material was frozen and thawed before filtration. 81 animals survived. No tumour was found in any of them.

#### 4. Serial Transfer of the Sarcomas in Mice

The mouse tumours were successfully transplanted to adult mice belonging to the same colony. Minced mouse tumour tissue was suspended in 4 parts of Hanks solution and 0.2 ml of the suspension



times contained fairly broad, bloodfilled vascular spaces lined with a hyperplastic endothelium (Fig 6) In the periphery the tumours infiltrated the fatty tissue, musculature and the skeleton In several areas mitoses were fairly numerous

The pulmonary metastases showed the same picture of spindle-cell sarcoma as the tumours at the site of inoculation (Fig 7) Some were of large-cell type, others of small-cell type The tumour metastases often formed perivascular cuffs around medium-sized vessels (Fig 8) An admixture of leucocytes was often seen in and around metastases Many animals had widespread pneumonic changes in the lungs

The tumour at the renal hilus had the character of a fairly highly differentiated fibrosarcoma This tumour might have been spontaneous Unfortunately no material from this tumour was set aside for further investigations

No metastases could be demonstrated in the retroperitoneal axillary, hilar or inguinal lymph nodes The lymph nodes often showed signs of non-specific inflammation

No metastases were seen in the liver or the spleen

### ■ *Effect of Age of the Mice*

The effect of the age of the mice is apparent from Table 1

The mice thus appeared to be susceptible only when inoculated within the first 2 days of life One animal developed a tumour after inoculation at 10 days of age Animals above 2 days were otherwise insusceptible

TABLE 1  
*Effect of Age of the Mice*

| Age at time of inoculation | Dose (ml suspension) | Mice with tumours surviving mice |
|----------------------------|----------------------|----------------------------------|
| 12 hours                   | 0.03-0.05            | 12/29                            |
| 1 day                      | 0.05                 | 3/22                             |
| 2 days                     | 0.05-0.1             | 6/13                             |
| 3-4 days                   | 0.05-0.1             | 0/6                              |
| 10 days                    | 0.15                 | 1/10                             |
| 13-20 days                 | 0.1-0.2              | 0/12                             |
| adult                      | 0.45                 | 0/15                             |

### 3 *Results of Injections of Chicken Tumour Extracts and Filtrates into Mice*

Extracts of the chicken sarcoma prepared as described above were injected subcutaneously into 3 litters of altogether 39 mice aged 12 hours to 4 days The dose injected varied between 0.05 and 0.15 ml according to the age of the animal Of these animals, of which 2 were lost by cannibalism, 2 developed tumours In one of the animals the tumour appeared at the site of inoculation after five and a half months

and grew progressively invading the thoracic wall and abdominal musculature. Histologically it was identical with the tumours described above. No gross or microscopic metastases were found. In the other animal the tumour appeared 6 months after inoculation, it grew slowly and had the histological character of a highly differentiated fibrosarcoma.

In addition a number of animals were inoculated with filtrates from the chicken sarcoma prepared in different ways. Two litters of eleven 2 day old mice were inoculated with filtrate prepared by centrifugation of a suspension of chicken tumour in an MSI centrifuge twice for 10 minutes at 4 000 r.p.m. after which the supernatant was filtered through a membrane filter AF 200 (Gottingen) (pore size  $1.3\mu$ ). Eight months later a hazel nut sized tumour with the histological characteristics of an adenocarcinoma developed in the right axilla of one of the animals. It had probably originated from the sweat glands or mammary. Attempts to transfer the tumour to chicken proved negative.

A litter of six 1 day old mice were inoculated daily with 0.03 ml of the same type of filtrate on 4 consecutive days and then with 0.1 ml on 2 days. No tumour developed (observation period 13 months).

Four litters of altogether 29 animals aged 12 hours to 4 days were inoculated with filtrate prepared in the above mentioned way with chicken tumour as starting material. This tumour had developed following injection of sarcoma suspension from a mouse inoculated with chicken tumour. Nineteen animals survived, in 3 of them tumours appeared in the thigh and in the back within 8-13 months and gradually assumed the size of walnuts and metastasized to the lungs and pleura. All the tumours had the histological character of an adenocarcinoma of the same type as that just mentioned. Inoculation of mice and chickens with these tumours gave negative results.

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Altogether 93 mice aged 12 hours to 7 days were inoculated subcutaneously with Seitz filtrate of a suspension of chicken tumours. In 5 mouse cases the tissue material was frozen and thawed before filtration. 81 animals survived. No tumour was found in any of them.

#### 4. Serial Transfer of the Sarcomas in Mice

The mouse tumours were successfully transplanted to adult mice belonging to the same colony. Minced mouse tumour tissue was suspended in 4 parts of Hanks solution and 0.2 ml of the suspension







were palpable in the breast musculature of the first 2 birds. The tumours grew slowly and at necropsy 4 weeks later large tumour conglomerates were seen in the thoracic wall. The tumour tissues were grey white sometimes mat and sometimes with a lustrous cut surface resembling that of a Rous sarcoma. The microscopic picture agreed also with that of the classical Rous sarcoma with bundles of spindle shaped cells separated by a myxomatous substance and free macrophage like cells (Fig 13). The chicken inoculated intraperitoneally died spontaneously after about 2 months with large intraperitoneal tumour masses resembling Rous sarcoma.

The tumour suspension prepared from a tumour in the first mouse passage induced tumour growth in one of the two chickens inoculated intramuscularly. Material from 13 different mouse passages was then injected into chickens the last injection being from the 52nd passage and gave rise to tumours in most birds (Fig 14).

Five inoculated chickens (from 4th 7th 9th 12th and 20th mouse passages) showed no tumour growth probably because the material had been taken from necrotic parts of the mouse tumour or because the chickens differed in susceptibility.

A cell free preparation of the mouse tumour was prepared by homogenizing tumour tissue in an Ultra Turrax (type 182 24 000 r.p.m.) for 10 half minute periods in an ice bath and collecting the supernatant after centrifuging in the Cold Centrifuge at 10 000 g for 20 minutes. Six chickens were inoculated into the breast muscle and 4 others with a similar preparation from mouse tumour grown in tissue culture for 9 days. No tumour developed.

Altogether 3 chickens were inoculated with Setz filtrate from freshly induced mouse tumours and from first passage tumours. In none of the birds did sarcoma appear.

Cell suspensions from the 2 mouse tumours observed after inoculation of chicken sarcoma extract were also inoculated into chickens. The one resembling the usual mouse sarcoma gave positive result while material from the fibrosarcoma proved negative. The latter tumour might thus have been a spontaneous one.

#### 6 Occurrence of Haemagglutinins in the Tumours and of Haemagglutination Inhibition in the Sera

In view of the possibility of contamination with polyoma virus the tumour tissue was tested for haemagglutinins. Material from 2 of the sarcomas induced in mice was tested for haemagglutinins in the way described by Ahlstrom & Jonsson (1962 b) for the rat tumour material. No haemagglutinins were demonstrable. The investigation was kindly performed by Dr Sven Bergman Department of Virology Lund.

Four adult mice were inoculated subcutaneously once a week for 3 weeks with 0.2 ml of a chicken tumour suspension in saline (1:10).



12



13

Figs 12-13

Fig 12 Metastasis in mesenterium of small intestine in the same mouse as in Fig 11. H&E stain  $\times 43$ .

Fig 13 Chicken sarcoma induced by alkalinising agent in mouse tumour (fifth passage). The usual picture of a Rous sarcoma. H&E stain  $\times 140$ .

on each occasion. One week after the third inoculation the sera from the mice were tested for polyoma antiviral antibodies by the haemagglutination inhibition test (Faddy *et al.* 1958). The titres were 1/30 to 1/240 and could be regarded as too low to indicate specific antibodies. The investigation was kindly performed by Dr H. O. Sjögren, Institute for Tumour Biology, Karolinska Institutet, Stockholm.

## DISCUSSION

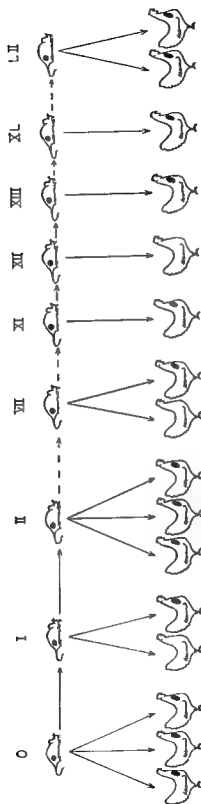
As in rats, hamsters and guinea pigs, material from the Schmidt Ruppin strain of Rous chicken sarcoma (originally deriving from the late Professor Ch. Oberling, Paris) induced sarcomas in mice. Cell suspensions from chicken sarcoma have thus evoked sarcomas in mice in a rather high frequency and in one case did a tumour occur in a mouse after inoculation with a presumably cell free supernatant of centrifuged chicken tumour material. Such material has however induced sarcomas in a much higher frequency in rats and hamsters. Attempts to induce tumours with Seitz filtrates in mice and in various other mammals proved unsuccessful owing probably to loss of virus during the filtration procedure. Cell free filtrates prepared in different other ways have given negative results in mice even after attempts to adapt the virus to mice or after passage in chicken fibroblast cultures. Similarly prepared filtrates have produced sarcomas in rats (Ahlstrom 1963). The tumours observed in a few of the mice were evidently spontaneous and could not be transferred back to chicken.

The chicken sarcoma thus seems to contain a virus, probably a variant of the Rous sarcoma virus with oncogenic capacity in mice.

This is in agreement with the findings of Zilber and collaborators (1962). With his strain Zilber induced not only sarcomas but also haemorrhagic cysts of the same appearance as those observed in this laboratory in rats but not in mice inoculated with the Schmidt Ruppin tumour strain. The surroundings of the mouse tumours induced by this strain however often contained wide distended vascular spaces. The differences can probably be explained by the use of different mouse and virus strains.

That the sarcomas in mice should represent heterotransplants of chicken tumour can be ruled out by various observations. Thus preliminary chromosome studies (Lévan 1962) showed that the mouse tumours were built up of mouse cells. Gel precipitation tests according to Wadsworth (1957) revealed no antigen common to mouse and chicken sarcoma (Skold 1963). The relatively long interval between inoculation and the appearance of tumours, progressive growth of the induced tumours and the possibility to transfer the tumour apparently indefinitely in unconditioned hosts argue definitely for the tumours being of mouse origin. These problems have been discussed in greater detail in earlier reports on experiments on the tumour inducing capacity





*Fig. 14*  
Diagram showing the results of back transfer to chicken of the Rous sarcoma in mice

tests, using anti-Rous sera are necessary to elucidate this problem. The negative results of the tests for haemagglutinins and haemagglutination inhibition tests and the lack of cross-reactions between polyoma-induced and Rous virus induced tumours in the transplantation experiments (Sjogren & Jonsson 1963) argue against contamination with polyomavirus. The mice inoculated with Rous virus never showed the spectrum of different tumours seen in animals inoculated with polyomavirus.

Pulmonary metastases were seen in a few animals with new-induced sarcoma. Whether the metastases were due to dissemination of tumour cells or to virus cannot be decided with certainty. The former possibility is supported e.g. by the fact that metastases were regularly much smaller than the tumours at the site of the inoculation.

The tumours could be carried in series in adult mice with cellular material already from the beginning, and from the 5th passage the frequency of takes was almost 100 per cent. The mice have been kept as a closed colony for several years and are probably genetically more homogeneous than the rats, in which the Rous virus induced tumours had to be carried initially in new-born animals. The adult mice, carrying passage tumours have, as a rule, shown no metastases, probably due to adaptation of the tumour to mice in which it grows quicker with consequent shorter survival of the hosts (Osterfeld 1941).

All attempts to transfer the tumour from mouse to mouse with different types of acellular preparations failed even when new-born animals were used. Inoculation of chickens with acellular material from mouse tumours also failed. The presence of virus in the mouse tumours has, however, been proved by the production of sarcomas in chicken following injection of a cell suspension also from the late mouse tumour passages, though the relatively long latency and slow growth of subsequent chicken tumours argue for a low virus content of the inoculate (Bryan 1946). The virus in the mouse tumour is in some way "masked" or the virus content of the cell free preparations is too low to elicit tumours. Similar results have been obtained with Rous virus induced tumours in rats, while infectious virus has been found in some hamster tumours (Svoboda *et al.* 1963).

The extreme variation in virus yield from Rous chicken sarcoma has been a problem ever since the original work with this tumour, and non-infective tumours passing through "nonfilterable phases" have frequently been found. One of the most important parameters determining the virus yield is the infective dose, small doses resulting in a larger fraction of non infective tumours (Bryan *et al.* 1955, Prince 1959). This explanation, however, appears less likely since the virus doses used in the mice were large. In the chicken, the virus yield does not seem to vary with the level of circulating antiviral antibodies (Prince 1959, Rubin 1962). Rubin assumes that the decrease in virus yield in the chicken tumour depends upon a cellular reaction of immunologically

of the Schmidt-Ruppin virus strain on rats, hamsters and guinea-pigs (Ahlstrom *et al* 1962 and 1963).

The findings are consistent with the susceptibility of mice to the virus being lower than that of rats, guinea-pigs and hamsters. Only 30 per cent of the surviving mice, inoculated with chicken tumour suspension, developed tumours, while the remaining kinds of animals studied showed a higher frequency of takes. The mice appeared mainly to be susceptible only when inoculated within the first 2 days of life, while rats developed tumours after inoculation up to the age of 23 days. The latency period in the mice varied widely, probably because of variations in the absolute size of the small inocula used for the mice resulted in a much greater relative difference in the size of the dose than did the corresponding variation in the absolute volume of the larger doses used for the other mammals.

The mouse thus shows the same resistance with increasing age against the oncogenic effect of the Rous virus as is known for other tumour-producing viruses, *e.g.* the polyoma-virus. Several explanations for this phenomenon may be considered. The tissues of adult animals might be resistant to tumour induction owing to an alteration in the target cells with increasing age. This point will be the subject of further investigation. In the polyoma system adult mice can be rendered susceptible by total body X-irradiation prior to virus inoculation (Dawe *et al* 1959), such irradiation probably interfering with the immunologic mechanism. Further, the susceptibility of suckling mice could be related to an induced immunological tolerance to the Rous virus. This point also requires attention. To return to the polyoma-system the immunological tolerance there does not seem to be of any importance because new born mice show good antibody-forming capacity after infection with polyomavirus (Sachs *et al* 1959, Rowe *et al* 1959) although the formation of antibodies is somewhat delayed.

Another observation of interest in the investigation of the resistance with increasing age is the demonstration of tumour-specific antigens in various forms of virus induced tumours, as revealed by transplantation studies. The existence of antigens common to various Rous-virus induced tumours in mice has recently been reported (Sjogren & Jonsson 1963), even though it could not be shown whether they are identical with viral antigen(s). In analogy with the mechanism suggested by Sjogren *et al* (1961) and Habel (1961) for polyomavirus it is possible that Rous virus inoculated into animals induces in infected cells the development of new antigens which are foreign to the host. The lack of tumour development in adult mice could then be due to the immunological response of the host animal to these antigens, while the growth of primary tumour cells in animals inoculated as new born could be explained by the development of tolerance to these antigens.

The possibility of the chicken sarcoma material being contaminated with some unknown oncogenic virus must be considered. Neutralization



competent cells in analogy with the homograft reaction to new cellular antigens, possibly identical with viral antigens. Such tumour-specific cellular antigens have been shown in Rous virus induced sarcomas in mice (Sjogren & Jonsson 1963), and if these should be identical with viral antigens, a similar reaction in the mouse should be considered as an explanation for the lack of infectious virus in the mouse tumours. Any discussion of the virus in the mouse cell cannot be more than speculative. The virus could exist in the form of a provirus and the tumour cell thus be "virogenic" (Livoff 1960).

Another explanation of the failure to find infectious virus in the mouse tumours can be based on Rubin's (1963) conclusion that Rous sarcoma virus is a defective virus, and that non-virus-producing cells need superinfection with Rous-associated virus (RAV) for the production of infectious Rous virus. The non-infective tumours in chicken after small virus doses could be explained by the solitary infection by defective Rous virus. *In vitro* studies by Temin (1962 and 1963) with cloned strains of Rous virus have shown that infected chick cells are more likely to give rise to converted, non-virus-producing (CNVP) foci, if they are grown with mouse cells than with chick cells. No virus could be found in the CNVP cells after freezing and thawing and as in the mouse tumour cells, no virus was detectable in tumour homogenates. Temin proposed that the mammalian tumours may contain Rous virus in the same state as the CNVP cells. An explanation for this state could be the unsuitable soil of the mouse cells *in vitro* and *in vivo* for the spread of RAV to the transformed and tumour cells, respectively.

The negative results of inoculations of mammals with highly virulent Rous sarcoma virus strains in this and other laboratories suggest that the Schmidt-Ruppin strain is a special variant rather than an especially virulent type. Preliminary serological studies point in this direction, too. Decision of this point requires further serological and immunological studies.

#### SUMMARY

After subcutaneous inoculation of a tumour suspension of Rous chicken sarcoma (Schmidt-Ruppin strain) into new born Swiss mice, 30 per cent of the surviving animals developed progressively growing sarcomas at the site of injection as a rule after 20-40 days. The sarcomas were of spindle cell type with an admixture of large polygonal cells. No cysts were observed. A few animals had lung metastases. Inoculation of cell-free filtrates gave negative results. Mice 13 days of age and older were resistant.

The sarcomas could be transplanted to adult mice from the beginning. Metastases were, as a rule, seen only after transplantation to new born animals. The sarcomas could not be transferred between mice with cell-free material.

The presence of virus in the mouse tumours was demonstrated by

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(Head Professor E. Waaler M.D.)

# VARIATIONS IN THE ONCOLYTIC ACTIVITY OF HUMAN SERUM

By

E. HARTVEIT

Received 14.1.64

The lytic action of human serum on cancer cells was first described by Freund & Hamner in 1910. Their observation was that while the serum of normal individuals was able to lyse human cancer cells, the serum of cancer patients was often unable to do so. That is to say they showed that human serum may be oncolytic to human cancer cells. In 1912 Kraus showed that human serum may also lyse mouse cancer cells. Since the advent of mouse ascitic tumours work on the oncolytic action of human serum has more or less been confined to its action on transplanted mouse ascitic tumours (see Hartveit 1964). As a result of this work all degrees of lytic ability have been ascribed to the serum from cancer patients. Serum from non-cancer patients has also been shown on occasion to lack lytic ability, while the serum from normal individuals is said to be uniformly lytic.

In view of this lack of conformity in different observations, results the oncolytic activity of a series of sera from patients with different diseases and from normal individuals was investigated.

## MATERIAL AND METHODS

Sera. 311 was obtained from patients in the wards and from 111 donors at Haukeland Hospital, Bergen. 154 of these were from the medical and nursing staff. Sera from 331 individuals were examined: 56 from 111 donors, 54 from cancer patients (111 surgically verified, 141 from medical and 113 from surgical non-cancer patients) and 26 from pregnant women. The serum was all used to elicit a room temperature (0-6°C) and then spun gently. The serum was decanted and stored at 4°C with no further processing.

at 4°C. Experiments were

Turner. The tumour is a

first needle at 16 days and

(Hartveit 1964). Sixty 8

Experiments were made. Tumour cell suspensions of one or two whole tumours were made up. One vol. of tumour cell suspension

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work which has

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# VARIATIONS IN THE ONCOLYTIC ACTIVITY OF HUMAN SERUM

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Received 14 VI 64

The lytic action of human serum on cancer cells was first described by Freund & Kaminer in 1910. Their observation was that while the serum of normal individuals was able to lyse human cancer cells, the serum of cancer patients was often unable to do so, that is to say they showed that human serum may be oncolytic to human cancer cells. In 1912 Kraus showed that human serum may also lyse mouse cancer cells. Since the advent of mouse ascitic tumours, work on the oncolytic action of human serum has, more or less, been confined to its action on transplanted mouse ascitic tumours (see Hartveit 1965). As a result of this work all degrees of lytic ability have been ascribed to the serum from cancer patients. Serum from non-cancer patients has also been shown, on occasion, to lack lytic ability, while the serum from normal individuals is said to be uniformly lytic.

In view of this lack of conformity in different observers' results the oncolytic activity of a series of sera from patients with different diseases and from normal individuals was investigated.

## MATERIAL AND METHODS

Sera. Blood was obtained from patients in the wards and from blood donors at Haukeland Hospital, Bergen, by courtesy of the medical and nursing staff. Sera from 391 individuals were examined: 56 from blood donors, 54 from cancer patients (histologically verified), 141 from medical and 113 from surgical non-cancer patients.

<sup>1</sup> Tumour

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**Experimental procedure.** Tumour cell suspensions of one vol. whole tumour ascites in 20 vol. physiological saline were made up. One vol. of tumour cell sus

<sup>1</sup> Research Cell

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ension was mixed with one vol. of serum. In control preparations saline was used in place of serum.

Wet preparations were made up from the mixtures and incubated in a moist chamber at 37° C for 30 min. after which the preparations were examined microscopically for cell lysis using a bright field condenser and cutting down the aperture.

The degree of lysis was recorded as follows—

|              |  |
|--------------|--|
| Non lytic    | no lytic cells present                 |
| Lytic        | both lytic and non lytic cells present |
| Highly lytic | only lytic cells present               |

Two tests using cell suspensions from different mice were carried out on each specimen.

## RESULTS

These are summarised in Table 1. All controls were negative i.e. non lytic.

TABLE 1

*The Oncolytic Ability of Fresh Human Serum from Normal Individuals (Blood Donors), Hospital Patients and Pregnant Women*

| Source of serum   | No. of cases | Oncolytic ability expressed as percentage |       |              |                 |
|-------------------|--------------|---|-------|--------------|-----------------|
|                   |              | No lytic                                  | Lytic | Highly lytic | Total oncolytic |
| Blood donors      | 51           | 77  | 3     | 14           | 23              |
| Cancer patients   | 54           | 47  | 11    | 30           | 53              |
| Medical patients  | 141          | 87  | 3     | 10           | 13              |
| Surgical patients | 113          | 60  | 24    | 16           | 40              |
| Pregnant women    | 27           | 48  | 11    | 11           | 5               |
| Total             | 391          | 70  | 10    | 14           | 30              |

see text

Of the total of 391 sera 70 per cent were non lytic, 16 per cent lytic and 14 per cent highly lytic. The repeat tests gave the same results. On further analysis differences between the lytic ability of sera from different groups of individuals were found. On combining the lytic and highly lytic sera into one group—oncolytic sera—in contrast to the non lytic sera it was found that the cancer patients and pregnant women show the highest percentage of oncolytic sera (53 and 52 per cent respectively) with surgical patients (40 per cent), next blood donors (23 per cent) and medical patients (13 per cent) last.

These variations were analysed further. I am indebted to the hospital staff for the access to clinical details.

*Blood donors.* No relationship was found between the lytic ability of the serum and the age, sex, ABO or rhesus blood group of the donor.

*Cancer patients.* No clear relationship to age or sex or to the extent of the disease was found. More material is needed before it is possible to say if the variations are related to the type of cancer.

*Medical and surgical non cancer patients.* The marked difference in

the percentage of lytic sera in these two groups is not related to age or sex. The only diagnosis that occurred frequently enough to allow any conclusion to be drawn was that of coronary artery disease. There were 34 such cases, 18 with recent symptoms of infarction. All but one of these patients had non lytic serum. Serum from the exception was lytic but not highly so. Repeat specimens gave the same result. The negative findings in this group can not, however, in themselves explain the excess of non lytic sera in medical compared to surgical patients.

*Pregnant women.* The lytic ability of the serum was not related to the duration of the pregnancy.

## DISCUSSION

The present findings show that there are differences in the oncolytic ability of serum from different groups of patients. The tests were carried out under controlled conditions (see *Hartvelt (A)*) and the results proved to be reproducible. The findings in the different groups can therefore be compared. They show that serum from cancer patients may be either lytic or non lytic, thus confirming both the previous positive and negative reports in the literature (*Bolande 1960 Kuru et al 1959*). Serum from pregnant women gave strangely similar results while medical non cancer patients—in particular patients with coronary artery disease—often showed non lytic serum. These findings can not as yet be explained.

The oncolytic activity of human serum on Ehrlich ascites carcinoma cells has been shown to be due to its complement content alone (*Hartvelt 1965 and (A)*) so at first sight these results appear to suggest a lack of correlation between the total serum complement level and malignant disease. On the other hand raised complement levels have been reported in cancer patients (*Fischel 1953*) and lowered levels in tumour bearing mice (*Hartvelt (C)*). So on second thoughts it becomes evident that the situation may not be so simple.

Recent work has shown that the Ehrlich ascites carcinoma cells are sensitized cells (*Hartvelt 1965 and (A)*). The antibody in this case has been supplied by the host mouse in response to the antigenic stimulus of the homografted tumour. It has also been shown that tumour cells may become sensitized in an otherwise genetically compatible mouse system (*Hartvelt (B)*). These cells, Bergen M ascites carcinoma cells, then behave in the same way as the Ehrlich ascites carcinoma cells—that is to say they lyse in the presence of fresh human serum and are capable of absorbing complement from such serum. This latter tumour host system is compatible genetically to that of the cancer patient and his tumour. Therefore the possibility that human cancer cells may also be sensitised must be considered. *Willheim & Iovs (1959)* finding that human cancer cells may lyse in the presence of fresh human serum supports this view as does *Freul & Kammer's (1910)* original observation.

If cancer cells are sensitized they can be expected to absorb complement *in vivo*. This would reduce the complement content of the patient's serum. If this serum were then tested against Ehrlich ascites carcinoma cells, it could be expected to be non-lytic but for one possibility. That is that the Ehrlich cells themselves could have absorbed complement as well as antibody *in vivo* (Hartvelt (C)). The only complement component that is present in any appreciable amounts in the mouse is C1 (Rice & Drowson 1950, McGhee 1952), so the cells could supply C1 to the system. If then C1 were the factor lacking in the patient's serum—as it might well be as it is the first factor used up by sensitised cells (Kabat & Meyer 1961), this could be supplied by the mouse, with the result that the patient's serum would appear to be oncolytic. This point, that the mouse could supply C1 to the oncolytic system, may be of importance in view of the suggestion made by Ginsberg *et al* (1961) that the C4 content of the sera of cancer patients may be reduced. Such sera should be non-lytic in this system.

The results in the blood donors show that normal human serum may have a wide range of oncolytic activity. This has not been stressed previously and is, at least, of practical importance. As there is such a wide range in the normal oncolytic activity of human serum, differences in disease will be difficult to interpret unless it can be shown, as suggested above, that there may be a difference not only between a positive and negative result, but also in the mechanism of the positive and negative results themselves.

#### SUMMARY

The lytic activity of 391 human sera was tested against Ehrlich ascites carcinoma cells. Both lytic and non-lytic sera were found in all groups examined, including blood donors. Cancer patients and pregnant women gave many highly lytic sera, while medical non-cancer patients—in particular those with coronary artery disease—gave few. It is suggested, on theoretical grounds, that it may be possible to analyse these differences further as the mechanism of the oncolytic reaction may differ in the different groups.

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Fig 1

Photomicrograph of the left mandible (H&E stain) Magnification  $\times 32$

left and the right buccal mucosa  
 adjacent to the palate close to the  
 adjacent a leucoplakia  
 mucosa opposite the lower  
 and the upper lip taken

All three biopsies were  
 and Mallory's staining  
 lengthened ulcers healed

of the parotid gland

the not extend beyond lamina

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## FORMATION OF VESICLES IN ORAL SUBMUCOUS FIBROSIS

By

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Received 9 vi 64

Oral submucous fibrosis has always been considered a disease of the connective tissue and very little attention has been paid to changes in the oral epithelium. A few authors (*Joshu 1953, DeSa 1957, and Sirsat & Khanolkar 1962*) described the occurrence of vesicles in the initial stages. The vesicles are mainly located to the buccal mucosa and palate. When the vesicles rupture, they turn into superficial ulcerations and heal by fibrosis. *DeSa* states that cultures of the vesicular fluid fail to reveal any specific organism.

So far, no reports are available on the histopathology of these vesicles. During an investigation of a number of patients with submucous fibrosis the present authors were able to remove vesicles in toto from two patients and examine these two vesicles histologically.

### CASE REPORTS

*Case 1*—Six months before examination a 22 year old man had blisters on his in the mouth and had difficulty in in without tobacco. At admission he soft palate. The mucosa in the palate the cheeks and anterior pillars had a blanched appearance and marked fibrous bands could be felt. One of the palatal vesicles was dissected under local anaesthesia.

The vesicle was cut in serial sections and the histological examination revealed the presence of a subepithelial vesicle (Fig 1). In a limited area the epithelium is lifted up by an accumulation of fluid. The epithelium covering the vesicle is atrophic and unkeratinized. The adjoining epithelium is slightly atrophic and the rete pegs have disappeared. Lamina propria is the seat of a rather marked fibrosis. In the juxtaepithelial zone there is a large number of thin walled vessels. The inflammatory reaction in the connective tissue below the vesicle is minimal. There are a few lymphocytes and polymorphonuclear leucocytes some of which are eosinophils.

*Case 2*—A 40 year old man had had no complaints regarding his mouth when he was examined. His submucous fibrosis was discovered incidentally. The patient has taken pan with tobacco and smoked 10 bidis daily for 25 years. The clinical examination revealed the presence of fibrous bands in the right buccal mucosa. Both the

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Requests for reprints should be addressed to J J Pindborg 4 Universitetsparken  
Copenhagen Denmark.  
Case 1 was examined at the Dental College Lucknow. The authors wish to thank  
Dr T A Chawla for cooperation.

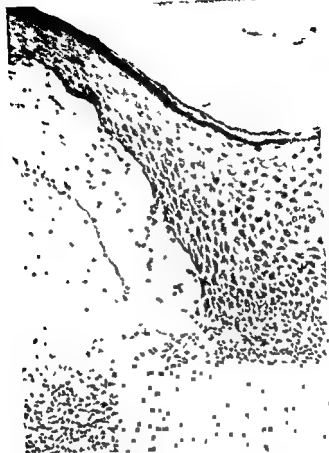


Fig 3

High power photomicrograph of area framed in Fig 2  
 Note the subepithelial nature of the vesicle Haematoxylin eosin  
 Magnification  $\times 320$

after Sircat & Khanolkar 1960, demonstrated changes similar to human submucous fibrosis by painting rat palates with capsaicin, the active irritant principle of chillies

#### SUMMARY

Two patients with oral submucous fibrosis are reported in whom vesicles were surgically removed. The histological examination revealed in both the cases occurrence of *subepithelial vesicles* associated with eosinophilic cells in the connective tissue.



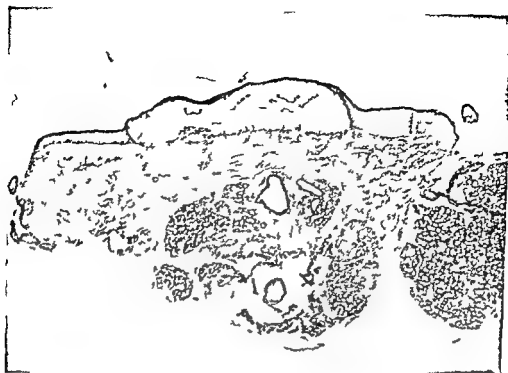


Fig 2

Low power photomicrograph of vesicle from Case 2. Haematoxylin and eosin

### DISCUSSION

In the two cases reported here the vesicles formed were undoubtedly subepithelial. No remnants of the basal cell layer of the epithelium were found on the connective tissue surface under the vesicles. Due to pressure from the underlying fluid the epithelium covering the vesicles was atrophic. In Case 1 the inflammation was minimal whereas it was marked in Case 2. In both patients a number of eosinophilic cells were observed.

Subepithelial vesicles or bullae of the oral mucosa are seen in the following conditions: pemphigoid, dermatitis herpetiformis, Duhring epidermolysis bullosa hereditaria, and bullous lichen planus. In erythema multiforme exudativum vesicles are observed intra- or subepithelial. Occurrence of many eosinophils may also be noticed in some of these diseases.

It would be tempting to assume an allergic reaction because of the eosinophilic cells in submucous fibrosis. As the disease almost exclusively occurs in Indians, a possible allergen should probably be found in the food. Sirsat (1958). As a number of patients has never been taking tobacco or betel, these habits are most likely not responsible for the development of submucous fibrosis. The histologic demonstration of subepithelial vesicles in submucous fibrosis should encourage further studies on a possible allergic nature of this condition and the more so

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## ISOZYME STUDIES OF SOME HUMAN CELL LINES<sup>1</sup>

By

I BECKMAN<sup>2</sup> and J D REGAN

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The use of human cell cultures for the study of human genetic traits not amenable to the classical methods of human genetics has for the last decade been a source of many interesting studies. Human cell culture genetics has certain limitations however the most serious of these being (1) the complexity of even the most minimal cell culture medium especially in that most cells require some amount of serum and (2) the limited number of genetic markers available.

Identification of the enzymes present in cultured cells and in particular the discrimination of multiple molecular forms of an enzyme isozymes (Markert & Moller 1959) by starch gel electrophoresis (Smithies 1959) makes available a class of genetic markers which have had relatively little use before.

Matz & DeCarli (1962) found that sub clones of the FUF strain differed considerably in their alkaline phosphatase activity. In some clones with low enzyme activity the alkaline phosphatase could be induced by cultivation in the presence of either prednisolone or phenyl phosphate. Preliminary results also indicated that in one sub clone there were at least three electrophoretically different alkaline phosphatase zones.

Paul & Fottrell (1961) described the esterase pattern in uncultured human cells in cultured human cells from skin kidney and heart and in HeLa cells. In all the cultured human cells there was a typical esterase pattern which was distinct from the pattern found in cultured mouse cells. There was essentially no difference between the esterase

activity (in aromatic ester) failed to give any results. The esterase zymogram patterns in cultured cells described by Komma (1963) are in close agreement with the findings by Paul & Fottrell.

<sup>1</sup> This investigation was supported in part by a grant from the Hawaii Division of the American Cancer Society to Dr J D Regan.

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Paul & Fottrell (1961) described the esterase pattern in uncultured human cells, in cultured human cells from skin, kidney and heart and in HeLa cells. In all the cultured human cells there was a typical esterase pattern which was distinct from the pattern found in cultured mouse cells. There was essentially no difference between the esterase pattern in fresh tissue and in cell lines grown for many years in culture, which suggests a high degree of genetic stability of the esterase enzymes.

Attempts to induce changes in the esterases by culturing them in the presence of a substrate (an aromatic ester) failed to give any results.

Kompa

ottrell

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<sup>1</sup> Contribution 31 of the Pacific Biomedical Research Center.

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Ueno & DeCarli (1962) found that sub-clones of the EURL strain differed considerably in their alkaline phosphatase activity. In some clones with low enzyme activity the alkaline phosphatase could be induced by cultivation in the presence of either prednisolone or phenyl phosphate. Preliminary results also indicated that in one sub-clone there were at least three electrophoretically different alkaline phosphatase zones.

Paul & Foltrell (1961) described the esterase pattern in uncultured human cells, in cultured human cells from skin, kidney and heart and in HeLa cells. In all the cultured human cells there was a typical esterase pattern which was distinct from the pattern found in cultured mouse cells. There was essentially no difference between the esterase pattern in fresh tissue and in cell lines grown for many years in culture, which suggests a high degree of genetic stability of the esterase enzymes. Attempts to induce changes in the esterases by culturing them in the presence of a substrate (an aromatic ester) failed to give any results. The esterase zymogram patterns in cultured cells described by Komma (1963) are in close agreement with the findings by Paul & Foltrell.

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<sup>1</sup> This investigation was supported in part by a grant from the Hawaiian Division of the

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## ISOZYME STUDIES OF SOME HUMAN CELL LINES<sup>1</sup>

By

I. BECKMAN\* and J. D. REGAN

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The use of human cell cultures for the study of human genetic traits not amenable to the classical methods of human genetics has for the last decade been a source of many interesting studies. Human cell culture genetics has certain limitations, however, the most serious of these being (1) the complexity of even the most minimal cell culture medium especially in that most cells require some amount of serum and (2) the limited number of genetic markers available.

Identification of the enzymes present in cultured cells and in particular the discrimination of multiple molecular forms of an enzyme isozymes (Markert & Voller 1959) by starch gel electrophoresis (Smithies 1959) makes available a class of genetic markers which have had relatively little use before.

Vaio & DeCarli (1962) found that sub-clones of the J U1 strain differed considerably in their alkaline phosphatase activity. In some clones with low enzyme activity the alkaline phosphatase could be induced by cultivation in the presence of either prednisolone or phenyl phosphate. Preliminary results also indicated that in one sub-clone there were at least three electrophoretically different alkaline phosphatase zones.

Paul & Foltrell (1961) described the esterase pattern in uncultured human cells in cultured human cells from skin, kidney and heart and in HeLa cells. In all the cultured human cells there was a typical esterase pattern which was distinct from the pattern found in cultured mouse cells. There was essentially no difference between the esterase pattern in fresh tissue and in cell lines grown for many years in culture, which suggests a high degree of genetic stability of the esterase enzymes. Attempts to induce changes in the esterases by culturing them in the presence of a substrate (an aromatic ester) failed to give any results. The esterase zymogram patterns in cultured cells described by Komma (1963) are in close agreement with the findings by Paul & Foltrell.

This investigation was supported in part by a grant from the Hawaii Division of the American Cancer Society to Dr J. D. Regan.

<sup>1</sup> Contribution 31 of the Pacific Biomedical Research Center.

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*De Carli et al* (1963) found a correlation between alkaline phosphatase activity and the presence of small acrocentric chromosomes. The finding of increased leucocyte alkaline phosphatase in Down's Syndrome patients (*Lennox et al* 1962) is of interest in this regard.

This is a report of isozyme studies on some continuous human cell lines and primary cells.

## MATERIALS AND METHODS

The cell lines studied were WISH amnion (*Hayflick* 1962), HeLa (*Gey et al* 1952), and RA a new line of human amnion (*Regan & Loh* data to be published). The WI 38 semi continuous cell strain (*Hayflick & Moorhead* 1961) was also examined. For comparison to primary tissue homogenates from three fresh amnions and from amnion epithelial cells cultivated for ten days *in vitro* were investigated as was human and calf serum. The cells were grown in 32 oz prescription bottles under standard conditions on Eagle's Basal Medium with 10 per cent inactivated calf serum.

Besides the control RA culture a number of RA cultures and in one case HeLa cells were grown in the presence of 10 µg/ml cortisone (11 dehydro 17 hydroxycorticosterone monoacetate) to test its effect on the zymogram patterns.

The cells were harvested by scraping them from the glass, washed twice in saline and homogenized in a minimal amount of fluid by a combination of mechanical treatment and freeze thawing. The homogenates were absorbed into small pieces of filter paper and subjected to starch gel electrophoresis. For the esterase and alkaline phosphatase zymograms the discontinuous buffer system by *Poulík* (1957) was used while for the studies of leucine aminopeptidase and catalase another discontinuous Tris buffer system was found to be more suitable (*Ashton & Braden* 1961). For the staining of esterases a naphthyl acetate was used with Blue RR salt as dye coupler. Alkaline phosphatase was stained with a naphthyl phosphate and Blue RR salt. Leucine aminopeptidase (LAP) was determined by the use of 1 leucyl a naphthyl

of the peroxide except in the areas where the peroxide had been destroyed by the catalase (cf *Paul & Foltz* 1961).

## RESULTS

Staining of the starch gels with Amido Black did not reveal any clearly defined protein zones in the homogenates from the cell lines. The samples rather appeared as diffuse columns clearly delineated from one another by the non staining space between them. The esterase zymograms revealed a pattern that agreed very well with the previous descriptions (*Paul & Iottrell* 1961, *Komma* 1963). There is a series of slow moving zones and one or two fast moving zones. The fast zones had a mobility close to that of the albumin associated esterase found in human serum (see Fig. 1). Essentially the same general pattern was found in primary amnion, the RA line, the WISH amnion line, the WI-38 strain, and the HeLa cells. Human serum has a quite different pattern. There were some minor variations between homogenates from different cultures. Thus in some harvests from the RA line there was only one fast zone instead of the usual two. We have also seen two cultures of RA cells with practically no trace of the series of slow bands but with two very strong fast bands.

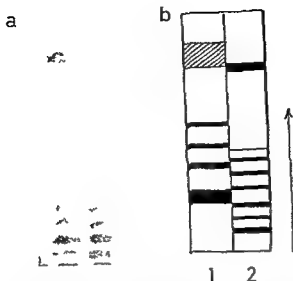


Fig 1

Esterase zymogram pattern in the RA amnion line a) photograph of starch gel, b) schematic picture showing human serum esterases (1) compared to the esterases of the RA cells (2) The arrow shows the direction of migration towards the anode

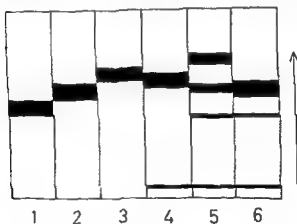


Fig 2

Schematic picture showing the alkaline phosphatase patterns in three different primary amnions (1-3) human serum (4) normal RA cells (5) and RA cells grown in the presence of cortisone (6) The four bands seen in sample 5 are called A B C and D in decreasing order of mobility towards the anode

Multiple alkaline phosphatase bands were observed in all three continuous cell lines studied (see Figs 2 and 3) The calf serum used in the medium did not show any alkaline phosphatase zone, nor did the WI 38 strain and the primary amnion epithelium cultivated for 10 days *in vitro* Extracts from fresh amnion showed only one enzyme band

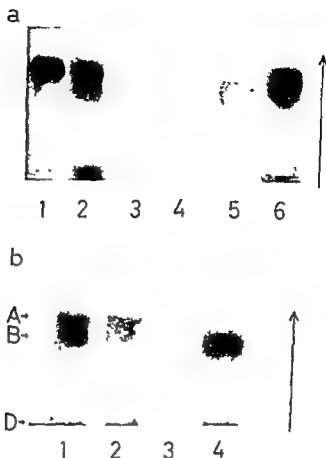


Fig 3

Photographs of starch gels showing alkaline phosphatase variations: a) HeLa cells (1) WISH amnion (2) two RA cultures showing weak alkaline phosphatase (3 and 4) human serum (5) and RA cells with strong fast band (6) b) Variations in the RA line Cells derived from survivors of 800 r (1) untreated culture (2) a subcloning of RA (3) and cortisone treated cells (4) Samples 1 and 2 show the A B and D zones 3 only the A zone and sample 4 has the B and D zones

which stained strongly. Three different amnions were studied and each of them had a distinct electrophoretic variant of alkaline phosphatase (Fig 2, samples 1-3). In the HeLa, WISH and RA lines we observed two distinct fast-moving zones (corresponding to zones A and B in Fig 2) and one slow-moving zone close to the start (zone D). Zone A of the cell lines was faster than the alkaline phosphatases of fresh human amnion and serum. The alkaline phosphatases of the RA line were followed continuously for six months in a series of 18 harvests of cultures. During the first month zone A was predominant, then for a period zone B became more pronounced and zone A weaker and in the last month of study zone A was stronger again and zone C more clearly visible than previously. A few RA cultures gave weak enzyme zones (Fig 3a, samples 3 and 4).

In 21 different cultures of RA cells grown in the presence of cortisone (10  $\mu\text{g}/\text{ml}$ ) and sampled over the whole period of study, the alkaline

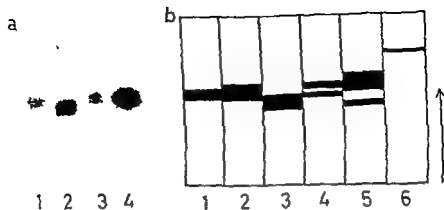


Fig 4

Variations in leucine aminopeptidase a) photograph showing calf serum (1), primary amnion (2) RA cells (3) and human serum (4). The extra fast moving zone in samples 1 and 4 is due to unspecific staining of serum albumin b) schematic picture showing calf serum (1) human serum (2) primary amnion (3) RA cells (4), WI-38 strain (5) and faint LAP band sometimes seen in RA and HeLa cells (6)

phosphatase zone A was absent, while zone B was apparently unaffected. In normal control cultures run in parallel zone A was present. Addition of cortisone to the harvested cells before homogenization did not alter the alkaline phosphatase pattern.

In human serum, calf serum and primary amnion a single, strongly staining zone of leucine aminopeptidase was found (Fig 4). The WI-38 strain (which lacked alkaline phosphatase) showed a clear and repeatable pattern consisting of two zones. RA cells cultivated under normal conditions showed usually no LAP activity. A faint fast moving zone (Fig 4, sample 6) was sometimes seen in RA and HeLa cells. In six different RA cultures grown in the presence of cortisone two distinct LAP bands were seen (Fig 4b, sample 4). These two enzyme zones are close together, can easily be seen when placing the starch gel on an X-ray box, but are difficult to visualize on a photograph.

Catalase activity was found in homogenates from all cultured cells and in primary amnion but not in calf serum. The mobility of the single catalase zone was rather variable. Cultures with slow or fast enzyme bands were found both in the RA line and the HeLa cells. It is not possible to ascribe any specific pattern to a certain cell line or tissue.

## DISCUSSION

Four different enzyme systems were detectable in human cells grown *in vitro*. It is of importance to clarify to what extent the enzyme activity found in the homogenate can be influenced by the addition of protein from the calf serum used in the medium. In the case of alkaline phosphatase and catalase no enzyme activity was found in calf serum and

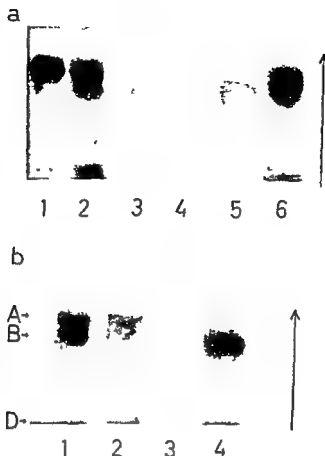


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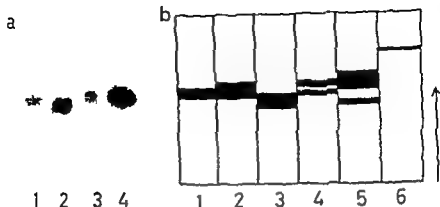


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#### DISCUSSION

Four different enzyme systems were detectable in human cells grown *in vitro*. It is of importance to clarify to what extent the enzyme activity found in the homogenate can be influenced by the addition of protein from the calf serum used in the medium. In the case of alkaline phosphatase and catalase no enzyme activity was found in calf serum and

the esterase and the leucine aminopeptidase enzymes were different from the ones found in serum. Hence, artefacts originating from the proteins of the medium can be excluded. The esterase patterns found were in good agreement with the findings by *Paul & Foltrell (1961)* and *Komma (1963)*. There are no striking variations between primary tissue and cell lines grown for many years, which suggests a high degree of stability of the esterase marker. On the other hand the fast esterase zones and the series of slow moving zones show strength variations, which, however, seem to be rather independent, *e.g.*, some cultures displayed very strong fast zones, while the series of slow esterase bands was staining weakly. This suggests that those two groups of esterases may be controlled by genes located on different chromosomes and that the variations are due to dynamic changes in the chromosomal stemline.

The strongly staining alkaline phosphatase enzymes in the primary amnions are probably derived from the deeper amnion layers (*Bourne 1962*). Trypsinization of amnion for primary cultures usually releases only the epithelial cells which show little or no alkaline phosphatase activity (cf. also *Fortelius 1963*). It is possible that the alteration into a continuous cell line is accompanied by the emergence of a series of different molecular forms of alkaline phosphatase. Of particular interest is the occurrence of a fast-moving zone in all three continuous cell lines, although the indistinguishable mobilities of the fast zones in the three lines is, of course, no proof of molecular identity. A whole series of electrophoretically different alkaline phosphatase enzymes have been identified in human serum (*Boyer 1961*). Three of the enzyme zones (by *Boyer* called A, B and D) occurred only in the sera of pregnant women and it was demonstrated that these enzymes were synthesized in the placenta. Another serum alkaline phosphatase zone has been found to be under the genetic control of at least three genetic loci two of which are the secretor and the ABO loci (*Beckman 1964*). Apparently a number of genes are controlling the human alkaline phosphatases and though essentially the same genetic information is present in every cell it is not always expressed. Thus in the placenta some particular alkaline phosphatases are synthesized (induced). The culture conditions may have caused an induction in the continuous cell lines of enzymes that are not normally synthesized in epitheloid tissue. The variability in the strength of the different alkaline phosphatases observed with time in these studies may possibly reflect dynamic changes in a heterogeneous cell population. In the RA line cortisone can apparently interfere in some way with the synthesis of the fast-moving alkaline phosphatase zone A. Zone B seems rather increased in cortisone treated cultures. Studies of clonal derivatives of the RA line and of survivors of  $\gamma$ -irradiation are in progress (see Fig 3b, samples 1 and 3). The RA clone seen in Fig 3b is interesting in that it shows only the zone A.

The results concerning the LAP variations in the RA line suggest that this cell line may be inducible for LAP, while *e.g.* the WI-38 strain is

constitutive for LAP. The mechanism of induction is not yet clarified. Clear LAP zones have been found only in six cortisone-treated cultures but most cortisone-treated cultures were negative. Studies of the mechanism of LAP induction are under way.

The electrophoretic variations of catalase were rather irregular and only a single blurred, wide zone was seen. Thus catalase does not seem to be a very informative enzyme marker in studies of cells cultivated *in vitro*.

#### SUMMARY

Electrophoretic variations in esterase, alkaline phosphatase, leucine aminopeptidase and catalase were studied in some human cell lines (HeLa, WISH amnion and RA amnion), the WI 38 strain, primary amnion and in serum.

There is little variation between the esterase patterns in fresh tissue and in cells cultivated *in vitro* for many years, suggesting a high degree of stability for the esterase enzymes. The RA cells show four different alkaline phosphatase components. They are apparently a rather delicate system, subject to considerable variation due to dynamic fluctuations in the population of cells. Cortisone is capable of interfering with the appearance of the fastest alkaline phosphatase zone when the cells are cultured in the presence of the hormone but not when the hormone is merely mixed with the homogenate. Two other continuous cell lines studied (HeLa and WISH amnion) had a zone electrophoretically indistinguishable from the fastest alkaline phosphatase of RA. The WI 38 cell strain and several samples of primary amnion both fresh and cultured briefly apparently did not possess this rapidly moving alkaline phosphatase.

Leucine aminopeptidase activity appeared weak and variable in the cell lines studied but was strong in the WI 38 cell strain showing two enzyme zones.

Catalase activity appeared as a wide blurred zone of variable electrophoretic mobility in the cell lines studied.

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## THE MUTAGENIC EFFECT OF HYDROXYLAMINE ON *ESCHERICHIA COLI*

By

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Hydroxylamine (HA) was first reported as a strong mutagenic agent by Freese and coworkers in 1961 (4). Working with phage T4, they found that the mutagenic effect was obtained only at high concentrations of HA and salt (NaCl) while low concentrations gave a rapid inactivation of the phage particles. Under optimal conditions as much as 5 per cent of the phages could be induced to mutate in the r-region of the phage chromosome.

HA attacks the base cytosine (1, 5, 8) and alters the structure of the base in such a way that it can pair with adenine instead of the usual guanine. During DNA replication this change would result in a base pair transition (5).

HA has been found to be an effective mutagen for animal viruses (11), tobacco mosaic virus (9) and for transforming DNA (6). It does also induce chromosome breakage in mammalian cells (10).

It is the purpose of the present paper to describe the effect of HA on the mutation rates in *E. coli*. In this connection an investigation of the gross inactivating effect was deemed relevant.

### MATERIALS AND METHODS

**Bacterial strains.** The test microbe used was the *E. coli* h 12 strain T 71 which has previously been used in this laboratory (7).

**Culture technique.** Heart infusion broth (HiB) and agar (both from Difco) were used as solid and liquid complete media. Minimal medium was that of Davis & Mingoli (2). Complete or minimal liquid media were incubated at 37°C. The cold (+4°C) was used for storage. A titre of approximately  $10^8$  cells/ml was used for all experiments.

**HA treatment.** The procedure was based on that described by Freese et al. (4).

**Hydroxylamine mixture.**  $\text{NH}_2\text{OH}\cdot\text{HCl}$  was dissolved in 2 X concentrated buffer. Such solutions were never kept for more than one week. Immediately before the addition of bacteria the pH was brought to 7.5 by means of concentrated NaOH.

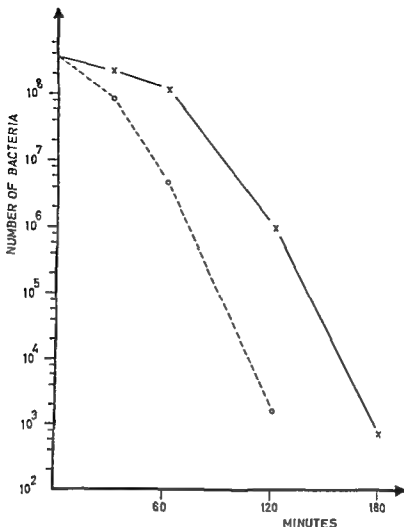


Fig 1

Inactivation curves of *E. coli* grown in complete broth when exposed to 1.0 M Hydroxylamine (—x—x—x—) and 0.1 M Hydroxylamine (---o---o---o---). Salt concentration in both cases 1.35 M NaCl

(12.5 M) NaCl was added to the desired concentration and pH again adjusted to 7.5. At zero time the bacterial suspension was added and incubation started in a 37°C waterbath.

**Genetic methods:** The inactivating effect of HA was analysed by determining the decrease in viable cell count employing a plate dilution method. In each determination the titre obtained represented the mean of the number of bacteria on at least three plates. Mutagenic effects were determined by an estimation of mutations from streptomycin sensitivity (str<sup>s</sup>) to streptomycin resistance (str<sup>r</sup>) and from dependence to non dependence of a particular growth factor. Details concerning these techniques have been described in the result section.

## RESULTS

### *The Inactivating Effect of Hydroxylamine*

In phage the inactivating effect of HA is greatest at low concentrations of the compound (4). From the experiment presented in Fig. 1 it is seen that the same is also true for cultures of *E. coli* in the stationary

growth phase. When the cells are exposed to 0.1 M HA the inactivating effect is significantly greater than when exposed to 1 M of the compound.

In the two curves presented the salt concentration is the same (1.35 M NaCl). This is taken to indicate that the concentration of HA is the main factor determining the rate of killing. When the salt concentration was decreased to 0.2 M NaCl, inactivation curves were obtained which were identical to those presented in Fig. 1. Thus, in contrast to the situation in phage (4) the salt concentration seems to be of little or no importance in determining the rate of killing in *E. coli*.

Successive series of experiments showed that concentrations of HA below 0.03 M were without significant inactivating effect in the technique used. On the other hand, concentrations above 3 M gave non-specific killing, presumably due to the high osmotic pressure of the reaction mixture.

In the experiments reported in Fig. 1, the cells were grown in complete media (H1B). Growth in minimal media regularly resulted in cells with greater resistance towards HA. With such cells the inactivation curves from separate experiments did not correspond so well. The highest concentration of HA, however, always gave the slowest inactivation rate.

### The Mutagenic Effect of Hydroxylamine

The mutagenic effect of HA on cells of *E. coli* was first studied in a system which employed the mutation from streptomycin sensitivity to streptomycin resistance. In the experiments presented in Table 1, cells were incubated in a reaction mixture which contained 1.2 M HA and 0.8 M NaCl. The zero time control consisted of cells and salt solution (1.5 M NaCl) only. After an exposure time such as indicated the cells were diluted 1:10 in stopping mixture, centrifuged and resuspended in saline to the original volume before plating on complete agar plates. After various hours of incubation, the agar was transferred to another agar plate of the same composition and volume which in addition contained 200 µg streptomycin per ml. After another 48 hours of incubation resistant colonies were counted.

TABLE 1

The Mutagenic Effect of Hydroxylamine (HA) on *E. coli* Number of Streptomycin Resistant Mutants per  $10^6$  Bacteria

| Incubation hours | Time of exposure to HA (min.) |     |      |
|------------------|-------------------------------|-----|------|
|                  | 0                             | 90  | 180  |
| 0                | 0                             | 0   | 0    |
| 3                | 3.2                           | 146 | 1786 |
| 5                | *                             | 263 | 2285 |

Reaction mixture: 1.2 M HA, 0.8 M NaCl. The numbers represent the mean of three plates.

\* Too heavy background growth of sensitive cells before exposure to streptomycin.

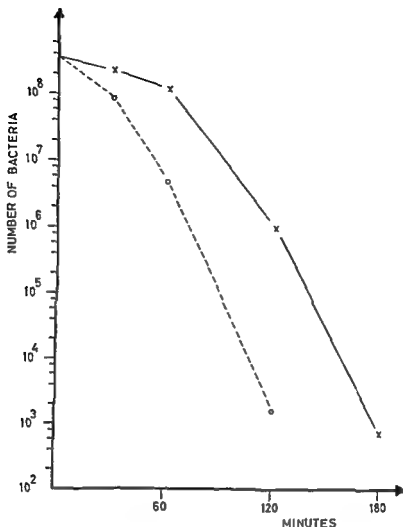


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phenomenon was first reported in phage (4) and has been found also in animal viruses (11). This paper shows that the same is true for bacteria. One explanation proposed (4) is that in a concentrated solution of HA there will be inactive ions present (e.g.  $\text{NH}_2\text{OH}^-$ ). Such ions may attach to the sensitive sites of the cell making these resistant to the action of HA.

The salt concentration of the reaction mixture does not influence the rate of bacterial killing, a phenomenon which is in contrast to the situation in phage. This could indicate that the main target of HA in bacteria is intracellularly located in a milieu of salt concentrations which are hardly influenced by that of the environment.

The inactivation rate increases with time of exposure to HA. Again this is analogous to the situation in phage (4). Purified RNA from tobacco mosaic virus, however, presents an exponential inactivation curve when exposed to HA (9). This would suggest that the alteration of a single base pair is sufficient for an inactivation of the whole molecule. The more complex inactivation curves obtained with phage and bacteria could therefore suggest that besides an inactivation due to reactions with DNA, HA may inactivate by reacting with some other cell constituents, most likely proteins.

HA exerts a great mutagenic effect on *E. coli*. Mutations are induced both from  $\text{str}^s$  to  $\text{str}^r$ , and from dependence to non-dependence of arginine in an arginine-requiring mutant. In both cases a period of active metabolism and replication is needed for the induced mutations to become expressed. The model of HA action would predict this, since the altered DNA-molecule must pass through two or more replications in order to get the base-transitions introduced. If great numbers of auxotrophic mutants were studied, only those which originally mutated by a transition from  $\frac{\text{A}}{\text{T}}$  to  $\frac{\text{G}}{\text{C}}$  should be found to revert upon treatment with HA.

Only one report has been found in which the mutagenic effect of HA on bacteria was examined (3). Eisenstark & Rosner studied the reversion pattern of 200 mutants in the *cysC* region of *Salmonella typhimurium* LT 2 using a series of different chemical mutagens. With HA no consistent positive results were obtained and the authors postulate that the substance is metabolized before it reaches the bacterial genome. The method used, however, is only briefly described, thus the cause of the discrepancy between their results and the positive mutagenic effect obtained in this paper remains uncertain.

#### SUMMARY

The inactivating and mutagenic effect of hydroxylamine (HA) on *E. coli* have been studied. The inactivation curves were complex with increasing inactivation rates with increasing time of exposure to HA.

The data presented in Table 1 show that HA does have a strong mutagenic effect on *E. coli*. It is also evident that this effect depends upon the length of exposure to the compound. It might be argued that the increased fraction of resistant cells is due to some kind of selective killing of sensitive cells. This cannot be the case, however, since the absolute number of str-r colonies after 90 minutes exposure to HA is far greater than the number in the untreated population.

When the concentration of HA is decreased to 0.1 M a definite mutagenic effect is still observed on this particular mutation in *E. coli*. In these experiments, however, the effect is more difficult to detect due to the more rapid killing which excludes an exposure time of more than 60 minutes. Variation of the salt concentration from 0.2 M to 1.3 M NaCl did not influence the mutagenic effects observed.

TABLE 2

*The Mutagenic Effect of Hydroxylamine (HA) on F. coli Mutation from Dependence to Non-Dependence of Arginine (arg)*

| Time of exposure<br>to HA (min) | Number of arg <sup>+</sup> cells per 10 <sup>6</sup><br>arg <sup>-</sup> cells when plated on |            |
|---------------------------------|---|------------|
|                                 | M   | M + HIB 1% |
| 0                               | 0   | 13         |
| 30                              | 18  | 160        |
| 90                              | 56  | 423        |

Reaction mixture: 1.0 M HA, 1.3 M NaCl. The numbers represent the mean of three plates.

In another series of experiments the mutation from dependence to non-dependence of a particular growth factor was studied. In the experiments presented in Table 2 an arginine-requiring mutant of the test microbe T 71 was grown in minimal medium supplemented with arginine for 25 hours. After treatment with HA the cells were plated on minimal media and on minimal media supplemented with 1 per cent broth (HIB). The number of residual divisions on this concentration of broth was determined by washing off the bacteria with saline at various time intervals and measuring the increase in viable cell count. With 1 per cent broth the number of residual divisions was found to be around five.

The data in Table 2 show that this strain of *E. coli* can be induced to revert to prototrophy upon treatment with HA. It is also evident that a great majority of the induced mutations needs replication or active metabolism in order to become expressed.

## DISCUSSION

One of the striking features of the inactivating effect of HA is the dependence of the inactivation rate on the concentration of HA. This

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# PULMONARY HYALINE MEMBRANES OF THE NEWBORN

*The Structure of the Membranes at Varying Postnatal Age*

By

BENGT ROBERTSON<sup>1</sup>

Received 29 ix 64

The relationship between hyaline membranes of the newborn and other pulmonary lesions, particularly intra-alveolar oedema, has been discussed by several authors (6-8, 10-13, 15, 17, 18). It has been maintained that the membranes are probably formed by a process of "condensation" of oedema fluid (12, 15, 17, 18). The presence of fibrin in the hyaline membranes is well consistent with this view (2, 5).

The appearance of the membranes has been found to vary with the postnatal age of the patient. It is generally agreed that the thickness of the membranes increases with the survival time (10, 13) though the inverse relationship has also been reported (9). A "macrophage response" has been described in cases surviving the first few days (4, 18). Proliferative and reparative phenomena in the alveolar walls proper have also been recognized in these cases (1). The purpose of this study was to examine the morphological features of pulmonary hyaline membranes of the newborn at varying postnatal age. The results have in part been presented in previous papers (14, 15).

## MATERIAL AND METHODS

The study was performed on 117 neonatal autopsy subjects, all of which had pulmonary hyaline membranes. The age of the patients varied between 6 hrs and 6 days. All cases had displayed clinical symptoms from the first day of life in the form either of respiratory distress or of episodes of cyanosis, multiple episodes of respiratory arrest or convulsions.

The term hyaline membrane is applied to the acidophil hyaline substance which lines the alveoli and/or alveolar ducts in a bandlike fashion. The

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the peripheral air-spaces (alveoli, alveolar ducts). For details concerning the grading system in this study we refer to the original paper published elsewhere (15). The presence of cellular infiltration in the membranes and the alveolar walls proper or proliferation of the alveolar epithelium was also recorded, however, without the application of a fixed grading system.

<sup>1</sup> Supported by grants from Karolinska Institutets reservatjonsanslag and Statens Medicinska Forskningsråd. Föreningen Sölsticket T 231



High concentrations of HA (1 M) allowed longer survival of the cells than low concentrations (0.1 M). The salt concentration (NaCl) did not influence the inactivation rates.

The mutagenic effect was studied using the mutations from streptomycin sensitivity to streptomycin resistance and from dependence to non-dependence of a particular growth factor (arginine). With high concentrations of HA a definite mutagenic effect could be detected in both cases. Variation of the salt concentration from 0.2 M to 1.3 M NaCl did not alter the mutagenic effect observed. Lower concentrations of HA also had a mutagenic effect although this was more difficult to detect due to the greater speed of inactivation.

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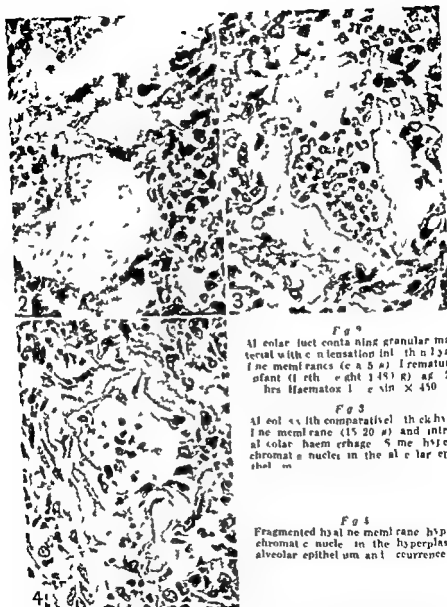


Fig 2

Alveolar duct containing granular material with condensation in the thin hyaline membranes (ca 5  $\mu$ ). Immature infant (10th month 145 g) age 21 hrs Haematoxylin-eosin  $\times 450$

Fig 3

Alveolar duct with comparatively thick hyaline membrane (15-20  $\mu$ ) and intra-alveolar haemorrhage. Some hyperchromatic nuclei in the alveolar epithelium

Fig 4

Fragmented hyaline membrane hyperchromatic nuclei in the hyperplastic alveolar epithelium and occurrence of

Cases existing during the third and fourth day displayed thick membranes (20-25  $\mu$ ) whereas intra-alveolar oedema was still less prominent than in the second group. Intra-alveolar haemorrhage and inflammation was a rather common finding. The alveolar epithelium beneath the membranes was often hyperplastic with hyperchromatic nuclei. The borderline between membranes and epithelium was in places indistinct (Fig 4). Particularly in the older ones of these cases the membranes

Histologic specimens from the lungs were examined in the following stains: haematoxylin-eosin, van Gieson and Lendrum's reticulum stain.

## RESULTS

The majority of the patients were premature and most of the infants, particularly the prematures, expired within the first 48 hours (Fig 1).

In cases dying within the first 24 hours, the hyaline membranes were generally thin ( $5-10\ \mu$ ), and intra-alveolar oedema was a prominent finding. Histologic pictures suggesting condensation of intra-alveolar oedema into thin hyaline membranes were often encountered in this age group (Fig 2). Inflammation or intra-alveolar haemorrhage were seldom present, nor was there any cellular infiltration in the hyaline membranes or the alveolar walls.

In patients who expired during the second day, the membranes were generally considerably thicker ( $15-20\ \mu$ ) and intra-alveolar oedema was not so prominent as in the former group. Still no cellular infiltration in the alveolar walls or the membranes was encountered, but in some places there were hyperchromatic nuclei in the alveolar epithelium. The borderline between membranes and alveolar walls was rather distinct (Fig 3).

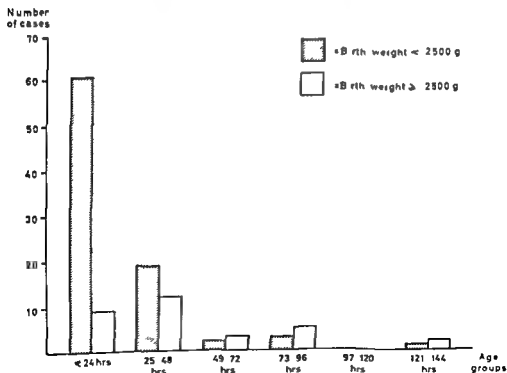


Fig 1

Distribution of the series (117 neonatal cases of pulmonary hyaline membranes: 68 males, 49 females) with respect to birth weight and age.

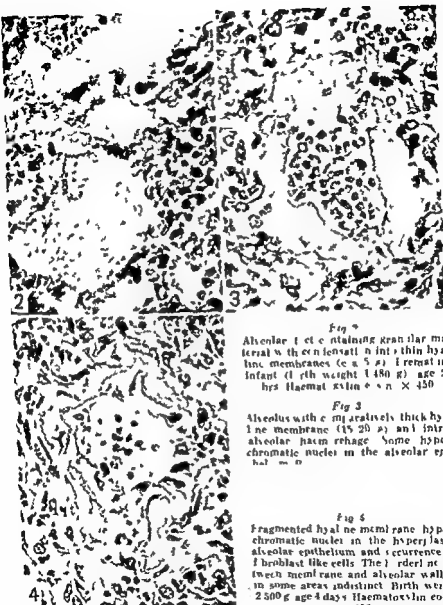


Fig 2

Alveolar tissue containing granular material with condensation into thin hyaline membranes (ca 5  $\mu$ ) (remains infant (1st weight 1480 g) age 21 hrs Haematoxylin & eosin  $\times 450$ )

Fig 3

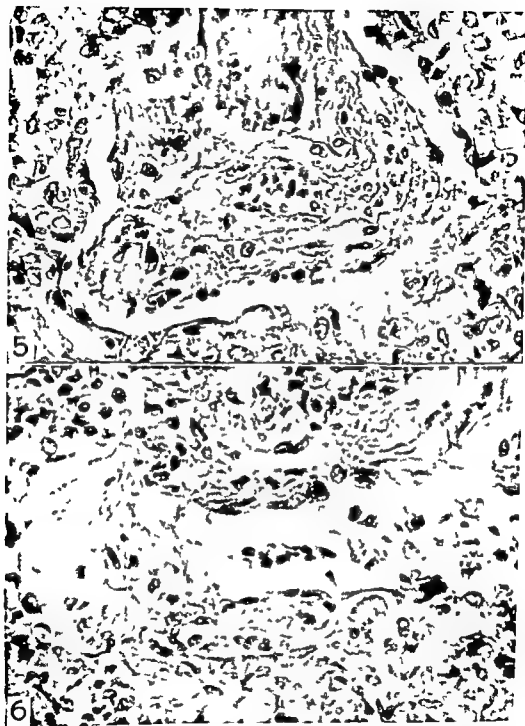
Alveolus with comparatively thick hyaline membrane (15-20  $\mu$ ) and intra-alveolar haemorrhage. Some hyperchromatic nuclei in the alveolar epithelium.

Fig 4

Fragmented hyaline membrane, hyperchromatic nuclei in the hyperplastic alveolar epithelium and occurrence of fibroblast-like cells. The borderline between membrane and alveolar wall is in some areas indistinct. Birth weight 2500 g age 4 days Haematoxylin eosin  $\times 450$

Cases expiring during the third and fourth day displayed thick membranes (20-25  $\mu$ ) whereas intra-alveolar oedema was still less prominent than in the second group. Intra-alveolar haemorrhage and inflammation was a rather common finding. The alveolar epithelium beneath the membranes was often hyperplastic with hyperchromatic nuclei. The borderline between membranes and epithelium was in places indistinct (Fig 4). Particularly in the older ones of these cases the membranes





Figs 5 &amp; 6

- Fig 5** Fragmented hyaline membrane infiltrated by histiocytes. The membrane is detached from the alveolar wall possibly by artefact. Birth weight 2 840 g, age 4 days. Haematoxylin & eosin  $\times 530$ .
- Fig 6** Alveolar duct with hyaline membrane infiltrated by histiocytes. The membrane merges with the wall of the duct. Birth weight 4 800 g (diabetic mother), age 6 days. Haematoxylin & eosin  $\times 420$ .

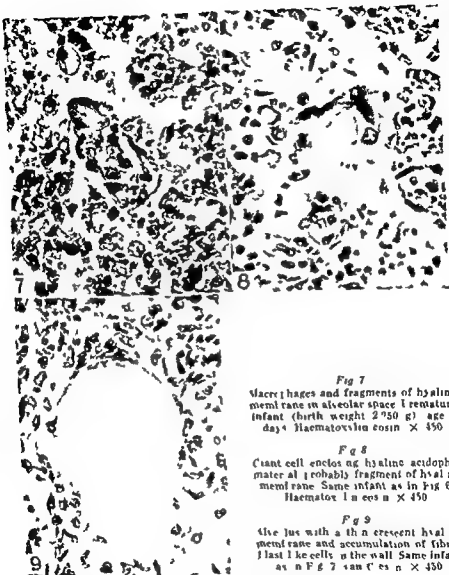


Fig 7

Macrophages and fragments of hyaline membrane in alveolar space. Termature infant (birth weight 2750 g) age 6 days. Haematoxylin-eosin  $\times 450$

Fig 8

Giant cell enclosing hyaline acidophilic material (probably fragment of hyaline membrane). Same infant as in Fig 6. Haematoxylin-eosin  $\times 450$

Fig 9

Alveoli with a thin crescent hyaline membrane and accumulation of fibroblast-like cells in the wall. Same infant as in Fig 7. Van Gieson  $\times 450$

were fragmented and infiltrated by histiocytes (Fig 5). In places the alveolar walls proper were thickened and showed excess of histiocytes and fibroblast-like cells (Fig 4). No excess of collagen or reticulation

was seen. In all patients displayed thick hyaline membranes (30-50  $\mu$ ) and practically no intra-alveolar oedema. In the third case the membranes were thinner (10-15  $\mu$ ) and there was moderate intra-alveolar oedema. In all these cases there was some degree of in-

flamination and intra alveolar haemorrhage. The hyaline membranes were largely detached from the alveolar walls and fragmented, but in places the membranes seemed to be partly incorporated in the alveolar walls (Figs 6-7). The hyaline material was infiltrated by many histiocytes and some giant-cells were present in the alveolar spaces. These giant-cells contained clumps of acidophil hyaline material, probably phagocytized fragments of hyaline membranes (Fig 8). As in the former age groups, the alveolar walls were often thickened and contained many histiocytes and fibroblast-like cells. There was no excess of collagen or reticulin fibers in the alveolar walls (Fig 9).

### COMMENT

The fact that the hyaline membranes tend to increase in thickness inversely with the degree of intra-alveolar oedema, suggests that the oedematous fluid "condenses" to form membranes, as has been pointed out earlier (12, 15, 17, 18).

The fragmentation of the membranes and the infiltration of these as well as of the alveolar walls by histiocytes and fibroblasts indicate a process of repair initiated on the third or fourth day of the disease. These observations, which agree with those of some other workers (4, 18), have been interpreted as evidence of a previous damage to the alveolar walls. It has been postulated that such damage probably is connected with the development of the hyaline membranes (1). Studies by the electron microscope have revealed destruction of the alveolar lining and the capillary walls in an early stage of the disease (2, 3). The cause of this destruction remains obscure but hypoxia may be a factor of importance. The hyaline membranes per se seem to be removed at least in part by macrophages and it is probable that many of these cells are derived from proliferating alveolar epithelium. The borderline between membranes and alveolar walls was often indistinct in cases surviving for more than three days. This feature suggests that the membranes, during the process of repair, to some extent become incorporated into the alveolar walls.

Similar features, i.e. hyperplasia of the alveolar epithelium, thickening of the alveolar walls with accumulation of histiocytes and fibroblasts, have been observed in patients with clinical histories suggesting hyaline membranes in the neonatal period, but dying at the age of 2-3 weeks of complications unrelated to their initial pulmonary disease (16). In these cases excess of collagen and reticulin fibers has been demonstrated in the thickened alveolar walls. These features have been interpreted as a later stage of such process of repair as described above.

Whatever the cause of this pulmonary disorder, it is evident that the hyaline membranes, when formed, initiate a "macrophage response" accompanied by reparative phenomena in the alveolar walls proper.

and it is probable that this process at least in some of the surviving cases may result in fibrosis of alveolar walls

### SUMMARY

The morphologic appearance of pulmonary hyaline membranes was studied in 177 neonatal autopsy cases, varying in age between 6 hrs and 6 days. The thickness of the membranes was found to increase with the survival time inversely with the degree of intra-alveolar oedema, which was a prominent finding in the cases dying at early stages. In cases surviving for three days or more, the membranes were found to be fragmented and infiltrated by histiocytes and giant-cells. Particularly in the six day-old cases the borderline between membranes and alveolar walls was often indistinct and the hyaline material seemed to some extent to be incorporated into the alveolar walls, which were thickened and infiltrated by histiocytes and fibroblast like cells. It is suggested that these features, which indicate a process of repair, may result in fibrosis of alveolar walls, as has been observed in patients with clinical evidence of hyaline membranes in the neonatal period but expiring later, at the age of 2-3 weeks.

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## OBSERVATIONS ON HUMAN COMPLETE AND INCOMPLETE 7S $\gamma$ -GLOBULIN ANTIBODIES AGAINST RED CELLS AND THEIR INTERACTIONS WITH THE RHEUMATOID FACTOR

By

K AHO, J LEIKOLA and K SIMONS

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We have previously studied the reaction of the rheumatoid factor (RF) with sheep erythrocytes sensitized with different subagglutinating doses of human 7S  $\gamma$ -globulin antibodies against sheep red cells, and with O Rh positive cells sensitized with incomplete anti-Rh antibodies (Aho, Harboe & Leikola 1964). The pattern of reaction of rheumatoid sera with sheep cells heavily sensitized with human anti-sheep cell antibodies and with O Rh positive cells sensitized with the rare "diagnostic" anti-Rh sera showed some basic similarities. Correspondingly, the system using sheep cells lightly sensitized with human anti-sheep cell antibodies and that using O Rh positive cells sensitized with anti-Rh sera suitable for Gm typing resembled each other. The purpose of the present work was to obtain additional information concerning this question by quantitating the  $\gamma$ -globulin fixed onto the cell surface under various experimental conditions.

### MATERIAL AND METHODS

*Immune sera.* The production of the human anti-sheep cell antibody sera has been described elsewhere (Aho, Harboe & Leikola 1964). The immune anti-AB sera were

... used with as were used. The rabbit anti-human  $\gamma$ -globulin serum No. 139 (Aho & Wager 1961) was used in the anti-globulin consumption tests.

*Fractionation of  $\gamma$ -globulin.* The serum to be fractionated was first dialyzed against 0.02 M phosphate buffer pH 8.0 whereafter it was transferred to a column

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... monoglobulins are  
...  $\gamma$ -globulin or briefly  $\gamma$ -globulin



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# OBSERVATIONS ON HUMAN COMPLETE AND INCOMPLETE 7S $\gamma$ GLOBULIN ANTIBODIES AGAINST RED CELLS AND THEIR INTERACTIONS WITH THE RHEUMATOID FACTOR

By

K AHO, J LEIKOLA and K SIMONS

Received 29 v 64

We have previously studied the reaction of the rheumatoid factor (RF) with sheep erythrocytes sensitized with different subagglutinating doses of human 7S  $\gamma$ -globulin antibodies against sheep red cells, and with O Rh positive cells sensitized with incomplete anti-Rh antibodies (Aho, Harboe & Leikola 1964). The pattern of reaction of rheumatoid sera with sheep cells heavily sensitized with human anti-sheep cell antibodies and with O Rh positive cells sensitized with the rare "diagnostic" anti-Rh sera showed some basic similarities. Correspondingly, the system using sheep cells lightly sensitized with human anti-sheep cell antibodies and that using O Rh positive cells sensitized with anti-Rh sera suitable for Gm typing resembled each other. The purpose of the present work was to obtain additional information concerning this question by quantitating the  $\gamma$ -globulin fixed onto the cell surface under various experimental conditions.

## MATERIAL AND METHODS

**Immune sera.** The production of the human anti sheep cell antibody sera has been described elsewhere (Aho, Harboe & Leikola 1964). The sera were produced by subcutaneous injection of 1 ml of sheep red cells rendered with Wiklund's and syngeneic sensitization of anti Gm12 (Aho et al.). From the sera, the IgG fraction was separated and dialyzed against distilled water. The IgG was then transferred to a column of Sepharose 4B and eluted with distilled water. The eluate was then transferred to a column of Sepharose 4B and eluted with distilled water.

The authors are highly indebted to Dr M Harboe of Oslo for his comments concerning the preparation of the manuscript and for valuable sera. In the present work the three major classes of serum immunoglobulins are termed  $\beta_1$  globulin,  $\beta_2$  globulin ( $\beta_2$  M) and 7S  $\gamma$  globulin or briefly  $\gamma$  globulin.

of Di Al cellulose equilibrated with the same buffer. Dilution with this same buffer gave a pure  $\gamma$ -globulin peak as shown by agar electrophoresis and immunoelectrophoresis. The effluent was concentrated by ultrafiltration. The  $\gamma$  globulin fractions were stored at  $-20^{\circ}\text{C}$ .

**Anti globulin consumption test** The test was performed by the dilution/absorption technique (Stiffen 1963). Appropriate amounts of cell suspensions usually corresponding to 0.1 ml of packed cells were incubated with the antisera for 90 minutes at  $37^{\circ}\text{C}$ . When different cells were used in the same experiment the cell concentrations were adjusted by their haemoglobin content. Human albumin solution was usually added to the  $\gamma$  globulin fractions of antisera to prevent non specific aggregation of  $\gamma$  globulin onto the cell surface. The sensitized cells were washed four times with a large volume of chilled phosphate buffered saline pH 7.4 and suspended in a 2 ml volume. From this six 0.25 ml aliquots were taken and mixed with equal volumes of the last six twofold dilutions of anti- $\gamma$  globulin serum preceding the final titre. After 15 minutes at  $37^{\circ}\text{C}$ , the cells were centrifuged and the supernates were separated and tested for the remaining anti  $\gamma$  globulin activity by a microscopic technique (Harboe & Inderall 1959). As a detector system heavily sensitized Rh positive cells were used. The consumption results were recorded in half dilution steps based on the strength of agglutination in the last reactive dilution. The basic consumption of the non sensitized cells was usually half a titre step.

## RESULTS

**Experiments with anti-Rh sera** Consumption experiments were performed with two diagnostic anti-Rh sera (anti-CD Ripley and anti-D Wiklinska) and with six other anti-Rh sera, three of which were suitable for the demonstration of anti-Gm(a). O Rh positive cells were sensitized with increasing amounts of each of the eight anti-Rh sera. The maximal consumption varied very little from serum to serum, although the amount of anti-Rh serum required for this maximal consumption displayed great differences. Four of these sera were titrated simultaneously and the results are shown in Table 1. It appears that the same amount of  $\gamma$ -globulin was fixed onto the cell surface from the "diagnostic" and from the other anti-Rh sera. Control experiments with the 7S  $\gamma$ -globulin fraction of one anti-Rh serum showed that the maximal consumption in this instance was not greater than that from the original anti-Rh serum.

TABLE 1  
Consumption of Anti  $\gamma$  Globulin Serum by Anti Rh Antibodies

| Volume in ml of anti Rh serum used for sensitizing 0.1 ml of packed cells | Anti $\gamma$ Globulin consumption titre |                  |                   |                 |
|---|--|------------------|-------------------|-----------------|
|   | Anti CD Ripley                           | Anti D Wiklinska | Anti CD Björkmark | Anti D Holm     |
| 0.8   |  |                  | 3                 | 3               |
| 0.4   |  | 3                | 2 $\frac{1}{2}$   | 3               |
| 0.2   | 3  | 3                | 2                 | 2 $\frac{1}{2}$ |
| 0.1   | 3  | 2 $\frac{1}{2}$  | 1 $\frac{1}{2}$   | 1 $\frac{1}{2}$ |
| 0.05  | 2 $\frac{1}{2}$                          | 2                | 1                 | 1               |
| 0.025   | 2 $\frac{1}{2}$                          | 1                | 1 $\frac{1}{2}$   | 1               |
| 0.012   | 1 $\frac{1}{2}$                          | 1                |                   |                 |
| 0.006   | 1  |                  |                   |                 |

not done

**Experiments with human 7S  $\gamma$  globulin antibodies against sheep red cells** Sheep erythrocytes were sensitized with increasing amounts of 7S  $\gamma$  globulin fractions from four persons (T P, P V, K V, and J L) and the anti globulin consumption titres were determined as above. The results with a simultaneous comparison with two anti Rh sera are shown in Table 2. It is seen that heavily sensitized sheep erythrocytes consumed markedly more of anti  $\gamma$  globulin serum than O Rh positive cells sensitized with an excess of anti Rh serum. The sensitizing dose corresponding to this maximal uptake in the Rh system was 1/16-1/32 of the minimum agglutinating dose (MAD).

TABLE 2

*Consumption of Anti  $\gamma$  Globulin Serum by Human 7S  $\gamma$  Globulin Antibodies against Sheep Red Cells*

| Cells sensitized with | Anti globulin consumption titre |     |     |     |
|-----------------------|---------------------------------|-----|-----|-----|
|                       | T P                             | P V | K V | J L |
| 1/2 MAD               | 6                               | 6   | 6   | 6   |
| 1/4 MAD               | 5                               | 6   | 5   | 5   |
| 1/8 MAD               | 4                               | 5   | 4½  | 4   |
| 1/16 MAD              | 3½                              | 4   | 3½  | 3½  |
| 1/32 MAD              | 2½                              | 3   | 2½  | 2½  |
| 1/64 MAD              | 1½                              | 2   | 2   | 1½  |

The maximal consumption of anti Rh sera Liljemark and Hallitu (CeDe cells) was 3½ and 3 titre steps respectively.

MAD: Minimum agglutinating dose.

TABLE 3

*Consumption of Anti  $\gamma$  Globulin Serum by Human 7S  $\gamma$  Globulin Antibodies against Group AB Cells*

| Cells sensitized with | Anti globulin consumption titre |     |
|-----------------------|---------------------------------|-----|
|                       | K V                             | L H |
| 1/2 MAD               | 5                               | 4   |
| 1/4 MAD               | 3½                              | 3   |
| 1/8 MAD               | 2½                              | 2   |
| 1/16 MAD              | 2                               | 1½  |

The maximal consumption of anti Rh sera Liljemark and Korpela (CeDe cells) was 3 and 2½ titre steps respectively.

MAD: Minimum agglutinating dose.

**Experiments with human 7S  $\gamma$  globulin antibodies against AB cells** The ability of the 7S  $\gamma$  globulin antibodies of K V and L H to render human group AB cells agglutinable by rheumatoid sera was at first studied. Agglutination experiments with selected rheumatoid and normal sera revealed that the reaction pattern closely resembled that previously seen when human 7S  $\gamma$  globulin antibodies against sheep red cells were used as sensitizing agents. The only difference observed was

that the anti-sheep cell system was slightly less dependent upon the sensitizing dose

Anti-globulin consumption experiments were then performed in a similar fashion as with the anti-sheep cell system. The results are collected in Table 3. It appears that the heavily sensitized group AB cells consumed more of the anti- $\gamma$ -globulin serum than the O Rh positive cells maximally sensitized with incomplete anti-Rh sera. The sensitizing dose corresponding to this uptake was  $1/4$ – $1/8$  MAD, i.e., somewhat less than that in the anti-sheep cell system.

## DISCUSSION

Evidence has accumulated indicating that the incomplete anti-Rh antibodies are of the 7S  $\gamma$  globulin type, whereas the saline agglutinating antibodies are of the  $\beta_2$ -macroglobulin type (e.g., Fudenberg, Kunkel & Franklin 1959). Recently, Greenbury, Moore & Nunn (1963) demonstrated that rabbit  $\beta_2$ M antibodies agglutinate red cells much more efficiently than 7S  $\gamma$ -globulin antibodies. The rare human Derythrocytes also are agglutinated by the incomplete anti-Rh antibodies (Race, Sanger & Selwyn 1951), suggesting that these erythrocytes contain more D receptors than the other D positive erythrocytes. It is possible that the agglutination is due to traces of saline agglutinating  $\beta_2$ M antibodies, although the observation gives a hint that the incompleteness of the 7S anti-Rh antibodies might be due to the limited number of available Rh receptors.

In the present work, anti-globulin consumption experiments were performed with cells sensitized with some human complete and in complete 7S  $\gamma$ -globulin antibodies. The recent experiments of Forsler (1963) have shown that this test can be considered a useful semiquantitative technique for the demonstration of bound  $\gamma$ -globulin. It was observed that sheep red cells and human group AB cells heavily sensitized with the 7S  $\gamma$ -globulin antibodies consumed more of the anti- $\gamma$ -globulin serum than Rh positive cells sensitized with an excess of anti-Rh serum. When the sheep cells or the AB cells were sensitized with an amount of  $\gamma$ -globulin corresponding to the maximal uptake in the anti-Rh system, the cells were not agglutinated and the antibodies thus were incomplete in the same manner as the 7S anti-Rh antibodies.

Our previous results (Aho, Harboe & Leilola 1964) suggested that the reaction of the RI<sup>+</sup> with sheep cells lightly sensitized with human anti-sheep cell antibodies is essentially an interaction of the RI<sup>+</sup> with individual  $\gamma$ -globulin molecules, whereas when heavily sensitized cells are used the reaction with immune aggregated  $\gamma$ -globulin plays a prominent rôle. The present experiments further emphasize the significance of the amount of sensitizing  $\gamma$ -globulin fixed on the cell surface. With heavily sensitized cells, the anti-sheep system and the anti-AB system could be used "diagnostically" for the demonstration of the RI<sup>+</sup>, and in these

instances the amount of  $\gamma$ -globulin fixed onto the cell surface was greater than in the anti-Rh system. It seems reasonable to assume that the limited number of available Rh receptors does not usually provide conditions for intermolecular interactions of the antibody molecules, and consequently this system is not usually suitable for the demonstration of those RF components reacting with immune aggregated  $\gamma$ -globulin.

The few 'diagnostic' anti Rh sera make an exception. Our previous results (Aho, Harboe & Leikola 1964) indicate that the Gm type is not crucial in determining whether an antiserum can be used "diagnostically", and it appears in the present work that the cells are not more heavily coated in the "diagnostic" than in the usual anti-Rh systems. Thus their unusual serologic behavior remains difficult to explain, although it may be speculated that the antibody molecules in these sera are more liable to configurational changes characteristic of the immune aggregated state than in other anti-Rh sera.

#### SUMMARY

Anti globulin consumption experiments were performed with cells sensitized with some incomplete and complete 7S  $\gamma$ -globulin antibodies. In the anti Rh system the consumption varied very little from serum to serum if an excess of anti Rh serum was used for sensitizing the cells. No difference was observed between the "diagnostic" and the other anti Rh sera. The maximal amount of antibody fixed onto the Rh positive cells corresponded to 1/16–1/32 MAD in the anti sheep cell system and to 1/4–1/8 MAD in the anti AB system. The results suggest that the limited number of available Rh receptors might be a sufficient explanation of the incompleteness of the anti-Rh antibodies.

The heavily sensitized (with 1/2 MAD) sheep red cells and group AB cells could be used 'diagnostically' for the demonstration of the RF, emphasizing the significance of the amount of sensitizing  $\gamma$  globulin fixed on the cell surface. However, the behavior of the rare "diagnostic" anti Rh sera could not be explained on this quantitative basis.

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# A NON-SPECIFIC INHIBITOR TO VACCINIA HAEMAGGLUTINATION IN POST MORTEM HUMAN SERA

By

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The vaccinia haemagglutination-inhibition (HI) test is known to be a valuable and convenient diagnostic aid, at least in the initial phase of a smallpox outbreak (3, 5, 8). Being extensively used during the smallpox outbreak in Stockholm in 1963, the usefulness of the HI test could be confirmed, on the whole, also by us (6).

During the latter outbreak a certain alarm was evoked by the incidental finding of a high HI titre in a serum sample drawn post mortem from an unvaccinated man, who had died suddenly from an acute illness with fever, purpura and an atypical rash.

It was soon discovered, however, that this high HI titre was due to a non specific inhibitor, and consequently a large number of post mortem sera were investigated.

The occurrence of such inhibitors is reported in this short communication. An account is also given of the possibility of removing or inactivating the inhibitors by pre-treatment of sera with different agents.

Non specific inhibitors to vaccinia haemagglutinin have been found previously in such materials as umbilical cord blood and ascites fluid (1). Szathmari *et al* described similar inhibitors in various pathological body fluids such as pleural exudate, fluid from hydrocele testis and ovarian cysts etc (11). Szathmari also found that kaolin absorption, and to a certain extent the action of trypsin, could remove these non specific inhibitors.

## MATERIAL AND METHODS

### *Vaccinia Haemagglutinating Antigen (HA Antigen)*

Bottle cultures of HFp 2  
cent calf serum were infect  
chick embryo passage. Aft  
vanced the liquid was rha  
After 2 days of further inc  
the supernatant saved as the HA antigen

= 1:1000 centrifuged and

### *Sera*

Forty five post mortem blood samples were obtained at autopsies performed 9 to 83 hours after death. All samples were taken at the same hospital and reached the



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within 9-12 hours reach a high titre which subsequently does not increase appreciably.

TABLE 1

*Distribution of HI Titres to Vaccinia Haemagglutinin in 45 Post Mortem Sera Titrated without Treatment and after Absorption with Kaolin*

|                          | Numbers of sera with the respective HI titres |    |    |    |    |     |     |     | Total number of sera |
|--------------------------|---|----|----|----|----|-----|-----|-----|----------------------|
|                          | 10  | 10 | 20 | 30 | 80 | 160 | 320 | 640 |                      |
| Untreated sera           | 2   | 3  | 2  | 8  | 10 | 11  | 4   | 5   | 45                   |
| Sera treated with kaolin | 41  | 2  | 1* |    | 1† |     |     |     | 45                   |

\* Fresh vaccinia pox.

† Vaccinated 1 month before death.

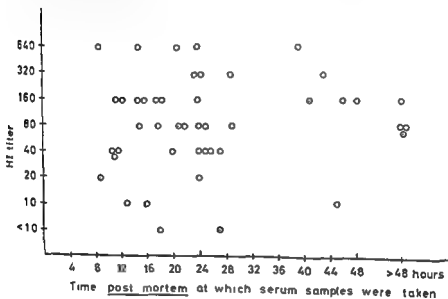


Fig. 1

*Inhibitory titres to vaccinia haemagglutinin in 45 post mortem sera drawn at different times after death*

### *Further Attempts to Remove the Non-Specific HI Titres*

The non-specific inhibition titres were not significantly affected by the routine heat inactivation. The action of four different methods of serum pre-treatment was studied in a comparative test. The agents tested were trypsin, chloroform, periodate and kaolin, which are known to remove effectively non specific inhibitors in other HI systems (*e.g.* myxovirus and arbo-virus HI tests). Fifteen post mortem sera and five positive and five negative sera were treated on the same day and then run in a simultaneous HI test.

laboratory within 4 hours. Serum was taken off immediately and then stored frozen at  $-25^{\circ}\text{C}$  until used.<sup>1</sup>

Positive normal control sera were obtained from verified smallpox cases. A number of prevaccination sera served as negative normal controls.

### *Serum Treatment*

All sera, even those termed 'untreated' in the sequel, were heated at  $60^{\circ}\text{C}$  for 20 minutes and then absorbed with one 10th volume of rooster erythrocytes.

The following methods were tested for their ability to remove or destroy the non-specific inhibitors present in post mortem sera.

*Kaolin treatment* (2). One volume of serum was mixed with four volumes of a '25 per cent' suspension of acid washed kaolin (Fisher Scientific Co. Fair Lawn N.J.). After 20 minutes at room temperature under occasional shaking the mixture was centrifuged at 2500 rpm for 15 minutes. The clear supernatant was used as serum diluted 1:5.

*Chloroform treatment* (9, 12). To 1 volume of serum were added 4 volumes of chloroform (analytic grade), the mixture was shaken for 2 minutes and then left for 30 minutes at room temperature. The top layer (extracted serum) separated by an opaque disc from the bottom chloroform layer, was pipetted off and centrifuged for 20 minutes at 5000 rpm. The supernatant was considered as undiluted serum.

*Periodate treatment* (7). Three volumes of a 0.011-M solution of potassium periodate ( $\text{KIO}_4$ ) in saline were added to one volume of serum and the mixture was left at room temperature for 20 minutes. The oxidation process was interrupted by addition of one volume of a 3 per cent glycerol solution.

*Trypsin treatment* (10). One volume of a 0.8 per cent trypsin solution (Trypsin 1-300, NBC) in phosphate buffered saline pH 8.2 was mixed with 2 volumes of serum. The mixture was kept in a water bath at  $56^{\circ}\text{C}$  for 30 minutes.

### *Haemagglutination Inhibition (HI) Test*

The HI test was performed in tubes with two fold serum dilutions, starting from 1:10 and a constant amount of antigen (4 HA units per tube). A 0.5 per cent suspension of sensitive rooster erythrocytes was added 10 minutes after the antigen. The tubes were shaken and kept for 1 hour at  $37^{\circ}\text{C}$ , after which bottom patterns were read. The HI titre expressed as the reciprocal of initial serum dilution was determined by the last tube showing complete inhibition of the haemagglutination.

## RESULTS

### *Level of Non-Specific Titres and the Time of their Occurrence*

Almost all of the 45 post mortem sera had appreciable HI titres when tested without prior treatment. High titres, i.e. 80 or more, were found in 30 out of the 45 sera. It is evident that such titres would be highly suspect during a smallpox outbreak if their non-specific nature were not recognized. However, treatment of sera with kaolin reduced the titres drastically. The distributions of HI titres in untreated and kaolin treated sera are shown in Table 1. The two significant titres remaining after kaolin absorption were probably specific as judged from vaccination histories.

Sera had been drawn at different times, i.e. from 9 to 83 hours after death. As seen in Fig. 1, the correlation is poor between the height of the non-specific titres and the time after death at which blood samples were taken. Evidently the inhibitors develop soon after death and,

<sup>1</sup> The authors are much indebted to Dr. Å. Hoberg, Södersjukhuset, Stockholm, for valuable help in collecting sera and certain clinical data.

treatment. The chemical nature of the inhibitor was not further investigated.

The titre of the inhibitor in haemagglutination-inhibition tests with vaccinia antigen was of the same magnitude as the specific titres found among smallpox cases. The recognition of the non-specific inhibitor is of practical importance in epidemic episodes where specimens from obscure cases of sudden death may be subjected to laboratory investigation.

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TABLE 2

*The Effect of Treating post mortem Sera with Various Agents to Remove Non Specific Inhibitory Activity against Vaccinia Haemagglutinin*

|                            |     | Vaccinia HI titres of sera untreated and after treatment with various agents |         |            |           |        |
|----------------------------|-----|--|---------|------------|-----------|--------|
|                            |     | Untreated  | Trypsin | Chloroform | Periodate | kaolin |
| <i>Post mortem sera</i>    | 1   | 80   | 80      | 10         | < 10      | < 10   |
|                            | 2   | 40   | 80      | 10         | < 10      | < 10   |
|                            | 3   | 80   | 320     | 20         | < 10      | < 10   |
|                            | 4   | 80   | 160     | 40         | 10        | < 10   |
|                            | 5   | 640  | 160     | 40         | 20        | 20     |
|                            | 6   | 160  | 160     | 20         | 10        | < 10   |
|                            | 7   | 320  | 160     | 10         | 10        | < 10   |
|                            | 8   | 640  | 320     | 80         | 160       | 80     |
|                            | 9   | 320  | 160     | 10         | < 10      | < 10   |
|                            | 10  | 640  | 640     | 20         | < 10      | < 10   |
|                            | 11  | 640  | 640     | < 10       | < 10      | < 10   |
|                            | 12  | 640  | 320     | 40         | < 10      | < 10   |
|                            | 13  | 320  | 320     | 10         | < 10      | < 10   |
|                            | 14  | 40   | 10      | < 10       | < 10      | < 10   |
|                            | 15  | 40   | 40      | 10         | 10        | < 10   |
| <i>Variola conial sera</i> | V 1 | 320  | 160     | 160        | 320       | 80     |
|                            | V 2 | 640  | 640     | 320        | 640       | 160    |
|                            | V 3 | 640  | 640     | 640        | 640       | 320    |
|                            | V 4 | 640  | 640     | 640        | 640       | 640    |
|                            | V 5 | 160  | 160     | 160        | 320       | 160    |
| <i>Neg human sera</i>      | N 1 | < 10   | < 10    | 10         | < 10      | < 10   |
|                            | N 2 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                            | N 3 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                            | N 4 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                            | N 5 | < 10   | < 10    | < 10       | < 10      | < 10   |

The effect of the various treatments is seen in Table 2. The negative control sera were unaffected by the treatments. The specific titres of positive sera were somewhat reduced by the kaolin absorption but not significantly changed by the other agents.

The HI titres of the post mortem sera could be reduced or completely removed by periodate and kaolin with approximately the same efficiency, whereas chloroform exhibited a marked but less complete effect. There was no unequivocal effect of the trypsin treatment.

#### CONCLUSION AND SUMMARY

The majority of post mortem sera were found to contain inhibitors to high titres against vaccinia haemagglutinin. The inhibitory activity was demonstrable as early as 9 hours after death. It could be removed by treatment with kaolin and periodate and partly removed through extraction with chloroform, but was virtually unaffected by trypsin.

treatment. The chemical nature of the inhibitor was not further investigated.

The titre of the inhibitor in haemagglutination-inhibition tests with vaccinia antigen was of the same magnitude as the specific titres found among smallpox cases. The recognition of the non-specific inhibitor is of practical importance in epidemic episodes where specimens from obscure cases of sudden death may be subjected to laboratory investigation.

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TABLE 2

*The Effect of Treating post mortem Sera with Various Agents to Remove Van Specific Inhibitory Activity against Vaccinia Haemagglutinin*

|                                  |     | Vaccinia HI titres of sera untreated and after treatment with various agents |         |            |           |        |
|----------------------------------|-----|--|---------|------------|-----------|--------|
|                                  |     | Untreated  | Trypsin | Chloroform | Periodate | Kaolin |
| <i>Post mortem sera</i>          | 1   | 80   | 80      | 10         | < 10      | < 10   |
|                                  | 2   | 40   | 80      | 10         | < 10      | < 10   |
|                                  | 3   | 80   | 320     | 20         | < 10      | < 10   |
|                                  | 4   | 80   | 160     | 40         | 10        | < 10   |
|                                  | 5   | 640  | 160     | 40         | 20        | 20     |
|                                  | 6   | 160  | 160     | 20         | 10        | < 10   |
|                                  | 7   | 320  | 160     | 10         | 10        | < 10   |
|                                  | 8   | 640  | 320     | 80         | 160       | 80     |
|                                  | 9   | 320  | 160     | 10         | < 10      | < 10   |
|                                  | 10  | 640  | 640     | 20         | < 10      | < 10   |
|                                  | 11  | 640  | 640     | < 10       | < 10      | < 10   |
|                                  | 12  | 640  | 320     | 40         | < 10      | < 10   |
|                                  | 13  | 320  | 320     | 10         | < 10      | < 10   |
|                                  | 14  | 40   | 10      | < 10       | < 10      | < 10   |
|                                  | 15  | 40   | 40      | 10         | 10        | < 10   |
| <i>Variola convalescent sera</i> | V 1 | 320  | 160     | 160        | 320       | 80     |
|                                  | V 2 | 640  | 640     | 320        | 640       | 160    |
|                                  | V 3 | 640  | 640     | 640        | 640       | 320    |
|                                  | V 4 | 640  | 640     | 640        | 640       | 640    |
|                                  | V 5 | 160  | 160     | 160        | 320       | 160    |
| <i>Neg human sera</i>            | N 1 | < 10   | < 10    | 10         | < 10      | < 10   |
|                                  | N 2 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                                  | N 3 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                                  | N 4 | < 10   | < 10    | < 10       | < 10      | < 10   |
|                                  | N 5 | < 10   | < 10    | < 10       | < 10      | < 10   |

The effect of the various treatments is seen in Table 2. The negative control sera were unaffected by the treatments. The specific titres of positive sera were somewhat reduced by the kaolin absorption but not significantly changed by the other agents.

The HI titres of the post mortem sera could be reduced or completely removed by periodate and kaolin with approximately the same efficiency, whereas chloroform exhibited a marked but less complete effect. There was no unequivocal effect of the trypsin treatment.

#### CONCLUSION AND SUMMARY

The majority of post mortem sera were found to contain inhibitors to high titres against vaccinia haemagglutinin. The inhibitory activity was demonstrable as early as 2 hours after death. It could be removed by treatment with kaolin and periodate and partly removed through extraction with chloroform, but was virtually unaffected by trypsin.

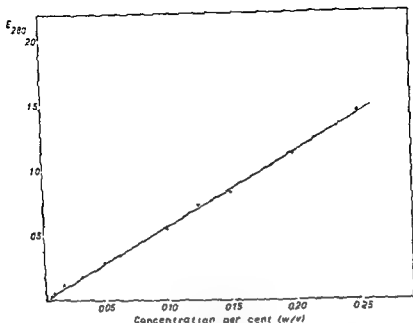


Fig 1

Calibration curve showing the extinction at 280 mμ in a spectrophotometer of different concentrations of β lipoprotein (obtained by dilution of the standard β lipoprotein solution)

#### Standard β Lipoprotein Solution

A 2 per cent solution (w/v) of human serum β lipoprotein prepared from whole serum (Op Nr 22263), was prepared by the method of Ingwerke AG Marburg of less than 1063. The standard solution was measured in a Beckman DU spectrophotometer using a 1 cm cell and a calibration curve for β lipoprotein was drawn (Fig 1).

#### Chromatographic Isolation of Human Serum β Lipoprotein

Hydroxylapatite was prepared as described by *Tiselius Hjerten & Levin* (1956). Hydroxylapatite columns with a height of 150 mm and a diameter of 10 mm were used.

The fractions were collected and their optical density at 280 mμ measured with a Beckman DU. Fig 1: The collection of β lipoprotein by the chromatographic technique all other fractions were discarded.



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## IMMUNOCHEMICAL STUDIES OF THE Lp(a) FACTOR

By

KÄRR BERG

Received 30 VII 64

The Lp system was described by Berg (1963). In gel diffusion tests (Ouchterlony 1958) absorbed rabbit immune sera distinguished genetic types of human serum  $\beta$ -lipoprotein. The  $\beta$  lipoprotein factor, demonstrated in this way, was called the Lp(a) factor. Individuals possessing this factor in their serum were said to be of type Lp(a+), those lacking it of type Lp(a—). The genetically determined Lp types are independent of previously known serum type and blood group systems (Berg & Mohr 1963, Mohr & Berg 1963, Berg 1964 a).

Antisera can be obtained by intravenous immunization of rabbits with either isolated  $\beta$  lipoprotein or whole human serum of type Lp(a+) (Berg 1965 a).

The reaction obtained with Lp(a+) human sera was weaker when the sera had been stored for several months at  $-25^{\circ}\text{C}$  (Berg 1963). It was therefore obviously necessary to study the stability of the Lp(a) factor.

In the present paper, further data on the characterisation and the stability of the Lp(a) factor are presented. The chromatographic method of Hjerten (1959), which the present author uses for purification of human  $\beta$ -lipoprotein, is also considered in this paper. The isolated  $\beta$ -lipoprotein, obtained by this technique, is useful for immunization of rabbits and further immunochemical study of the Lp(a) factor.

### MATERIALS AND METHODS

#### Human Sera

7 of type Lp(a+) and 13 of type Lp(a—) from several of the experiments. In addition sera from individuals involved in paternity cases and from

— cannot not include in the panel were used

All blood samples (Berg 1963), and the sera were stored at  $-25^{\circ}\text{C}$  until used

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I am indebted to all colleagues who have provided such sera

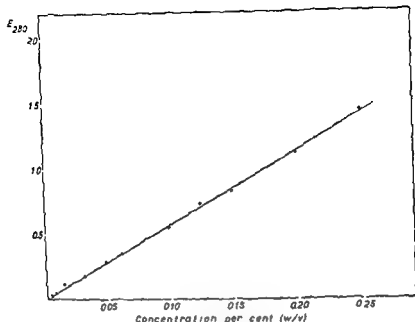


Fig 1

Calibration curve showing the extinction at 280 m $\mu$  in a spectrophotometer of different concentrations of  $\beta$  lipoprotein (obtained by dilution of the standard  $\beta$  lipoprotein solution)

#### Standard $\beta$ Lipoprotein Solution

The standard  $\beta$  lipoprotein solution was prepared from whole serum (No. 22263), was prepared by AG, Warburg. The concentration of the standard solution was 0.13 per cent (w/v). The solution was caused in a Beckman DU spectrophotometer using a 1 cm cell and a calibration curve for  $\beta$  lipoprotein was drawn (Fig 1).

#### Chromatographic Isolation of Human Serum $\beta$ Lipoprotein

The human serum  $\beta$  lipoprotein was isolated by the method of ...

Fig 1. The ...

The volume of the sample was 1.8 ml and the average optical density  $E_{280}$  was 2.355 corresponding to a concentration of 0.13 per cent (w/v). For the immunization

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## IMMUNOCHEMICAL STUDIES OF THE Lp(a) FACTOR

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The Lp system was described by Berg (1963). In gel diffusion tests (Ouchterlony 1958) absorbed rabbit immune sera distinguished genetic types of human serum  $\beta$ -lipoprotein. The  $\beta$ -lipoprotein factor, demonstrated in this way, was called the Lp(a) factor. Individuals possessing this factor in their serum were said to be of type Lp(a+), those lacking it of type Lp(a—). The genetically determined Lp types are independent of previously known serum type and blood group systems (Berg & Mohr 1963, Mohr & Berg 1963, Berg 1964 a).

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### MATERIALS AND METHODS

#### Human Sera

A panel of 20 normal human sera: 7 of type Lp(a+) and 13 of type Lp(a—) from laboratory personnel was used for several of the experiments. In addition sera from blood donors from unrelated individuals involved in paternity cases and from personnel not included in the panel were used.

All blood samples were used as such (Berg 1963), and the sera were stored at  $-25^{\circ}\text{C}$  until used.

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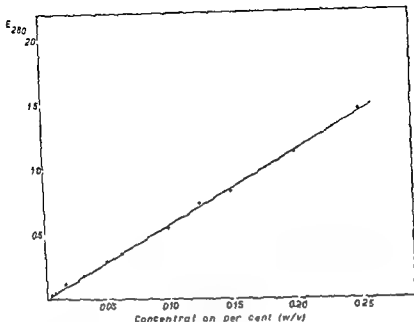


Fig 1

Calibration curve showing the extinction at 280 mμ in a spectrophotometer of different concentrations of β lipoprotein (obtained by dilution of the standard β lipoprotein solution)

#### Standard β Lipoprotein Solution

A 0.1 per cent solution (w/v) of human serum β lipoprotein prepared from whole serum by flotation in the ultracentrifuge at a density of 1.063 (Op. Nr. 29263) was kindly put at my disposal by Drs. Heide and Storko, Behringwerke AG, Marburg. This solution thus contained the lipoproteins with a density of less than 1.063. The optical density at 280 mμ of different dilutions of this standard solution was measured in a Beckman DU spectrophotometer using a 1 cm cell and a calibration curve for β lipoprotein was drawn (Fig. 1).

#### Chromatographic Isolation of Human Serum β Lipoprotein

Hydroxylapatite was prepared as described by Tiselius, Hjertén & Lelin (1956). Hydroxylapatite columns with a height of 150 mm and a diameter of 13 mm were used. The column was equilibrated with 0.05 M Tris buffer, pH 8.0.

Effluent was collected in 5 ml fractions and their optical density at 280 mμ measured with a Beckman DU spectrophotometer using a 1 cm cell (see Berg 1963, Fig. 1). The collection of β lipoprotein was however made by visual control on a 1 cm cell.

The β lipoprotein fraction from 85 ml of human serum was dialysed for 24 hours against four changes of one litre 0.85 per cent saline solution at 4°C. After the dialysis the average volume was 58 ml and the average optical density  $E_{280} = 2.35$  corresponding to a concentration of 0.43 per cent (w/v). For the immunization

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## IMMUNOCHEMICAL STUDIES OF THE Lp(a) FACTOR

By

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Received 30 VII 64

The Lp system was described by Berg (1963). In gel diffusion tests (Ouchterlony 1958) absorbed rabbit immune sera distinguished genetic types of human serum  $\beta$ -lipoprotein. The  $\beta$  lipoprotein factor, demonstrated in this way, was called the Lp(a) factor. Individuals possessing this factor in their serum were said to be of type Lp(a+), those lacking it of type Lp(a-). The genetically determined Lp types are independent of previously known serum type and blood group systems (Berg & Mohr 1963, Mohr & Berg 1963, Berg 1964 a).

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In the present paper, further data on the characterisation and the stability of the Lp(a) factor are presented. The chromatographic method of Hjerten (1959), which the present author uses for purification of human  $\beta$ -lipoprotein, is also considered in this paper. The isolated  $\beta$ -lipoprotein, obtained by this technique, is useful for immunization of rabbits and further immunochemical study of the Lp(a) factor.

### MATERIALS AND METHODS

#### Human Sera

A panel of 20 normal human sera, 7 of type Lp(a+) and 13 of type Lp(a-), from laboratory personnel was used for several of the experiments. In addition sera from blood donors, from unrelated individuals involved in paternity cases and from personnel not included in the panel were used.

All blood sera used (Berg 1963), and the sera were stored at  $-25^{\circ}\text{C}$  until used.

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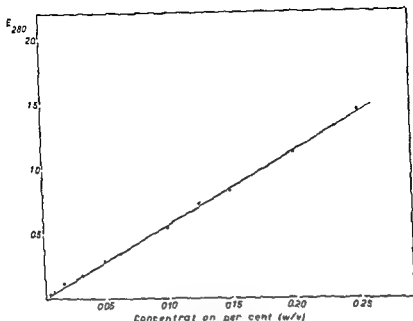


Fig. 1

Calibration curve showing the extinction at 280 m $\mu$  in a spectrophotometer of different concentrations of  $\beta$  lipoprotein (obtained by dilution of the standard  $\beta$  lipoprotein solution)

#### Standard $\beta$ Lipoprotein Solution

A 2 per cent serum by fluid kindly put This solution optical density measured in curve for  $\beta$  lipoprotein was used for the calibration

#### Chromatographic Isolation of Human Serum $\beta$ Lipoprotein

Hydroxylapatite was prepared as described by Tiselius Hjertén & Levin (1956). Hydroxylapatite columns with a height of 150 mm and a diameter of 12 mm were used for the chromatography. The columns were equilibrated with 0.10 M phosphate buffer of pH 6.8 and 8.5 ml of fresh undialysed human serum was applied to each column. The elution was performed as described by Hjertén (1959) using phosphate buffers of pH 6.8 of increasing molarity in the order 0.10, 0.20 and 0.65 M. The effluent was collected in 5 ml fractions and their optical density at 280 m $\mu$  measured with a Beckman DU spectrophotometer using a 1 cm cell (see Berg 1963, Fig. 1). The collection of  $\beta$  lipoprotein was however made by visual control in order to obtain the greater part of it in one batch (see Berg 1963).

concentration of 45 per cent (w/v) for the immunization

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## IMMUNOCHEMICAL STUDIES OF THE Lp(a) FACTOR

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Received 30 VIII 64

The Lp system was described by Berg (1963). In gel diffusion tests (Ouchterlony 1958) absorbed rabbit immune sera distinguished genetic types of human serum  $\beta$ -lipoprotein. The  $\beta$ -lipoprotein factor, demonstrated in this way, was called the Lp(a) factor. Individuals possessing this factor in their serum were said to be of type Lp(a+), those lacking it of type Lp(a-). The genetically determined Lp types are independent of previously known serum type and blood group systems (Berg & Mohr 1963, Mohr & Berg 1963, Berg 1964 a).

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### MATERIALS AND METHODS

#### Human Sera

A panel of 20 normal human sera, 7 of type Lp(a+) and 13 of type Lp(a-), from laboratory personnel was used for several of the experiments. In addition sera from blood donors, from unrelated individuals involved in paternity cases and from laboratory personnel not included in the panel were used.

Sera from 35 patients, suffering from different conditions requiring multiple blood transfusions, were used in one experiment. The patients had each received at least 5 blood transfusions. The sera were obtained from different clinical departments.<sup>1</sup>

All blood samples were obtained by venepuncture, treated as described elsewhere (Berg 1963), and the sera were stored at  $-25^{\circ}\text{C}$  until used.

---

<sup>1</sup> I am indebted to all colleagues who have provided such sera.

### Double Diffusion Tests in Agar Gel

The tests were performed either in Petri dishes filled with 25 ml. or on glass slides covered with 2.5 ml agar. The gel contained 1 per cent (w/v) agar, 0.85 per cent (w/v) NaCl, 10 per cent (v/v) 0.15 M sodium phosphate buffer of pH 7.0 and 1/1000 (w/v) merthiolate. Dishes and slides were equally good for the Lp tests (Berg 1964b).

Six peripheral wells and one central well were cut in the agar, the diameter of each well being 4 mm and the distances between neighbouring wells in all cases being 5 mm. The slides and dishes were kept in a moist chamber at 37°C. Readings of precipitates were made against a dark background by oblique illumination from below after 1, 2, 3 and 7 days. Visible precipitate was recorded as positive reaction, no visible precipitate as negative reaction.

### Immunoelectrophoresis

Immunoelectrophoresis was performed as described by Grabar & Williams (1953) with the micromodification of Scheidegger (1955).

### Staining

The precipitates in the gel diffusion tests were stained according to the method of Lief (1960) using either Oil Red O as a lipid stain or Amido Black 10 B as a protein stain (both from G. T. Gurr Ltd, London). The presence of esterase activity in precipitates was demonstrated as described by Lief (1961) using  $\beta$ -naphthyl acetate (L. Light & Co. Ltd, Colnbrook, England) and Diazo Blue B (G. T. Gurr Ltd, London).

### Photographic Registrations

The dishes and the slides were photographed in the moist unstained condition in indirect illumination. A Leica camera and 35 mm Arapex Agfa film were used.

## EXPERIMENTS AND RESULTS

### Characterization of the Lp(a) Factor as a Component of $\beta$ Lipoprotein of Lp(a+) Sera

The Lp(a) factor can, at present, only be studied by means of specific anti-Lp(a)-serum. Lp(a+) and Lp(a-) human sera (5  $\mu$ l of each) were submitted to immunoelectrophoresis, and the antiserum trough was filled with a specific anti Lp(a)-serum (100  $\mu$ l). Lp(a+) human sera gave one single precipitate, while sera of type Lp(a-) gave no precipitate. The precipitate corresponded to that of  $\beta$ -lipoprotein of human serum, with respect to the length of electrophoretic migration, the distance from the antibody trough, and the shape of the precipitation arc (Fig. 2).

It was found necessary to use as much as 5  $\mu$ l Lp(a+) human serum to obtain visible precipitates. This might indicate that the Lp(a) factor is a comparatively small part of the total  $\beta$ -lipoprotein. Different dilutions of human serum were therefore tested against unabsorbed rabbit immune serum. A human serum was diluted 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160 with a 0.85 per cent saline solution. 5  $\mu$ l of each dilution was submitted to immunoelectrophoresis. An unabsorbed sample of the immune serum used in the experiment described above was diluted with an equal part of 0.85 per cent saline solution. This gave the same dilution effect as the absorption of the same antiserum for use as anti-Lp(a) serum. Each of the antibody troughs was filled with 100  $\mu$ l of



of rabbits the different  $\beta$  lipoprotein hatches were adjusted to give an extinction as close to this value as possible

The optical density was usually measured in 1/10 dilutions of the  $\beta$  lipoprotein fractions and the concentration of  $\beta$  lipoprotein was read from the calibration curve. There is probably no major objection to the use of a solution of lipoproteins of density less than 1.063 prepared in the ultracentrifuge as a standard for the  $\beta$  lipoprotein fraction obtained by the chromatographic method as Cramer (1962) has found that the latter contains lipoproteins with densities from 1.063 to less than 1.006. No further quantitative studies of the  $\beta$  lipoprotein were within the scope of the present study.

#### *Preparation of Lipoproteins in the Ultracentrifuge*

Ultracentrifugation was carried out in the 40 rotor of a Spinco model I preparative ultracentrifuge<sup>1</sup> at 105 000 G for 24 hours. From each of the sera of 2 Lp(a+) and 2 Lp(a-) individuals lipoproteins of densities < 1.006, < 1.019 and < 1.063 respectively were prepared as described by Hatel Eder & Braidon (1955).

#### *Rabbit Anti Human Serum Antiserum*

Anti Humanscrum (Behringwerke AG Marburg Op Nr 470 I) was used as anti serum in the immunoelectrophoretic tests of serum protein fractions.

#### *Specific Rabbit Anti Human $\beta$ Lipoprotein Antiserum*

Anti  $\alpha_2$  Lipoproteinserum<sup>2</sup> (Behringwerke AG Marburg Op Nr 320 D) was used.

#### *Anti Lp(a) Sera*

Sera from rabbit No 1 absorbed 2/3 from No 3 absorbed 2/5 and from No 52 absorbed 2/3 with Lp(a-) human serum were used.

Sera from rabbit No 3 and No 52 were also used in the unabsorbed state for some of the experiments.

#### *Dialysis Bags*

Cellophane Cusings from Visking Co Chicago Ill were used.

#### *Concentration of Chromatographic Fractions*

Concentration was performed by dialysis against Polyethylene glycol 20 000 (Hoechst) as described by Kohn (1959).

#### *Petri Dishes*

ANUMBRA dishes with a diameter of 7 cm were used.

#### *Glass Slides*

Washed and polished 5 × 5 cm glass slides (Menzel Glaser) were used.

#### *Agar*

Rheinagar (Behringwerke AG Marburg Op Nr 18 141 142 147 148 150) and Bacto Agar (Difco Laboratories Detroit 1 Mich Control No 452925) were used. The Bacto Agar was washed and stored as described by Hirschfeld (1960) before use. For the Lp tests the first agar is just as good as the second and the only reason for using different sorts of agar was that one was more easily available than the other at the time of the investigations.

<sup>1</sup> I am indebted to Dr P Bjørnstad Institute of Clinical Biochemistry University of Oslo for permission to use the ultracentrifuge.

<sup>2</sup> The designation of this antiserum refers to the behaviour of the  $\beta$  lipoprotein in agar gel immunoelectrophoresis.

### Double Diffusion Tests in Agar Gel

The tests were performed either in Petri dishes filled with 24 ml. or on glass slides covered with 2.5 ml agar. The gel contained 1 per cent (w/v) agar, 0.85 per cent (w/v) NaCl, 10 per cent (v/v) 1/15 M sodium phosphate buffer of pH 7.0 and 110 000 (w/v) merthiolate. Dishes and slides were equally good for the I<sub>p</sub> tests (Berry 1964b).

Six peripheral wells and one central well were cut in the agar, the diameter of each well being 4 mm and the distances between neighbouring wells in all cases being 5 mm. The slides and dishes were kept in a moist chamber at 37° C. Readings of precipitates were made against a dark background by oblique illumination from below after 1, 2, 3 and 7 days. Visible precipitate was recorded as positive reaction, no visible precipitate as negative reaction.

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Immunoelectrophoresis was performed as described by Grubar & Williams (1953) with the micromodification of Scheidegger (1955).

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The precipitates in the gel diffusion tests of Uriei (1960) using either Oil Red O as a protein stain (both from G. T. Gurr Ltd, London) in precipitates was demonstrated as described acetate (L. Light & Co. Ltd, Colnbrook, Engl. London).

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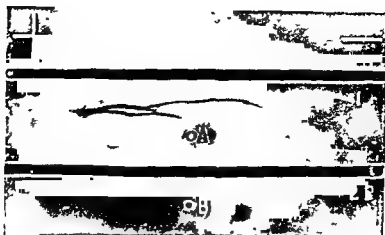


Fig 2

Immunoelectrophoretic characterisation of the Lp(a) factor. The reagents were

- A Human serum of type Lp(a+)
- B Human serum of type Lp(a-)
- 1 Rabbit-anti human serum-antiserum
- 2 Anti Lp(a) serum

A precipitate corresponding to the  $\beta$  lipoprotein of the Lp(a+) serum is found against the anti-Lp(a) serum whereas no precipitate can be seen between the Lp(a-) serum and the antiserum

this diluted, unabsorbed rabbit immune serum. A distinct precipitate was observed corresponding to the  $\beta$ -lipoprotein of the serum portions diluted 1/40 or less, whereas no precipitate could be seen where the human serum had been diluted 1/80 or more.

In double diffusion tests,  $\beta$ -lipoprotein fractions, as well as fractions from the other steps of the chromatography, were tested against anti Lp(a)-serum. A precipitate developed between the antiserum and  $\beta$ -lipoprotein from Lp(a+) individuals when the concentration of the fraction was 20–25 per cent (w/v) or higher. No precipitate was formed between the antiserum and fractions from the other steps of the chromatography, or between the antiserum and  $\beta$  lipoprotein of type Lp(a-), even when the fraction had a considerably higher concentration than mentioned above.

Lipoproteins of densities  $< 1.006$ ,  $< 1.019$  and  $< 1.063$ , prepared in the ultracentrifuge from Lp(a+) and Lp(a-) individuals, were tested against anti-Lp(a)-serum. A precipitate was found only against the lipoprotein of density  $< 1.063$  from Lp(a+) individuals. From this it was concluded that the Lp(a) factor belongs to the 1.019–1.063 density class of human serum lipoproteins.

Lp(a+) human serum was dialysed against equal parts of a 0.85 per cent saline solution for 24 hours. The serum and the dialysate were thereafter tested against anti Lp(a)-serum, and it was found that the Lp(a+) serum still reacted with the antiserum, whereas no reaction was observed between the antiserum and the dialysate. It was concluded that the Lp(a) factor is not removed from whole human serum by

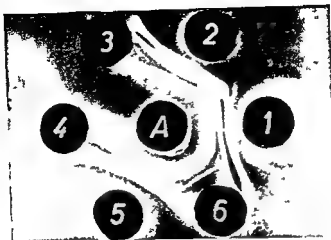


Fig 3

Spur formation between a  $Lp(a+)$  and a  $Lp(a-)$  human serum (wells 1 and 2)  
The reagents were:

- A Unabsorbed rabbit immune serum
- 1 Human serum of type  $Lp(a+)$
- 2 and 3 Human serum of type  $Lp(a-)$
- 3 and 4 0.8% per cent saline solution
- 5 Anti  $Lp(a)$  serum

dialysis. Several of the  $\beta$  lipoprotein batches were also tested before the dialysis described in the section dealing with chromatographic isolation of this protein. The test results were always identical to those obtained after dialysis, indicating that this procedure had no major damaging effect on the  $Lp(a)$  factor.

All precipitates between anti  $Lp(a)$  serum and  $Lp(a+)$  human serum, or between the antiserum and solutions of isolated  $Lp(a+)$   $\beta$  lipoprotein, could be stained by protein and lipid dyes, and the presence of esterase could be demonstrated. The  $Lp(a)$  factor is therefore a lipoprotein possessing esterase activity.

$Lp(a+)$  and  $Lp(a-)$  human sera were tested in double diffusion experiments against unabsorbed rabbit immune sera. When the immune sera contained anti  $Lp(a)$  antibody, a spur could usually be seen between the precipitates formed by  $Lp(a+)$  and  $Lp(a-)$  human sera, indicating that the  $Lp(a+)$  serum possessed at least one antigenic component more than the  $Lp(a-)$  sera (Fig 3).

In some experiments, an additional precipitate near the well containing  $Lp(a+)$  serum developed instead of a spur (Fig 4).

The additional line of precipitation showed a reaction of identity with the precipitate obtained with anti  $Lp(a)$  serum. This experiment demonstrated that the  $Lp(a)$  factor is a separate antigenic component, different from the rest of the  $\beta$  lipoprotein. The additional precipitate observed in some experiments, might even indicate that the  $Lp(a)$  factor is found in a separate group of  $\beta$  lipoprotein molecules.



Fig 2

Immunoelectrophoretic characterisation of the Lp(a) factor The reagents were

- A Human serum of type Lp(a+)
- B Human serum of type Lp(a-)
- 1 Rabbit-anti human serum antiserum
- 2 Anti Lp(a) serum

A precipitate corresponding to the  $\beta$  lipoprotein of the Lp(a+) serum is found against the anti Lp(a) serum whereas no precipitate can be seen between the Lp(a-) serum and the antiserum

this diluted, unabsorbed rabbit immune serum A distinct precipitate was observed corresponding to the  $\beta$ -lipoprotein of the serum portions diluted 1/40 or less, whereas no precipitate could be seen where the human serum had been diluted 1/80 or more

In double diffusion tests,  $\beta$  lipoprotein fractions, as well as fractions from the other steps of the chromatography, were tested against anti-Lp(a)-serum A precipitate developed between the antiserum and  $\beta$ -lipoprotein from Lp(a+) individuals when the concentration of the fraction was 0.20-0.25 per cent (w/v) or higher No precipitate was formed between the antiserum and fractions from the other steps of the chromatography, or between the antiserum and  $\beta$ -lipoprotein of type Lp(a-), even when the fraction had a considerably higher concentration than mentioned above

Lipoproteins of densities < 1.006, < 1.019 and < 1.063, prepared in the ultracentrifuge from Lp(a+) and Lp(a-) individuals, were tested against anti-Lp(a)-serum A precipitate was found only against the lipoprotein of density < 1.063 from Lp(a+) individuals From this it was concluded that the Lp(a) factor belongs to the 1.019-1.063 density class of human serum lipoproteins

Lp(a+) human serum was dialysed against equal parts of a 0.85 per cent saline solution for 24 hours The serum and the dialysate were thereafter tested against anti Lp(a) serum, and it was found that the Lp(a+) serum still reacted with the antiserum, whereas no reaction was observed between the antiserum and the dialysate It was concluded that the Lp(a) factor is not removed from whole human serum by

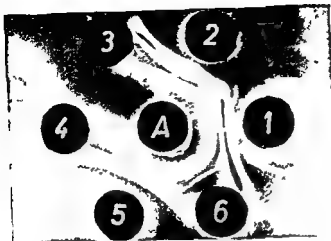


Fig 3

Spur formation between a Lp(a+) and a Lp(a-) human serum (wells 1 and 2)  
The reagents were

- A Unabsorbed rabbit immune serum
- 1 Human serum of type Lp(a+)
- 2 and 5 Human serum of type Lp(a-)
- 3 and 4 0.85 per cent saline solution
- 6 Anti Lp(a)-serum

dialysis. Several of the  $\beta$ -lipoprotein hatches were also tested before the dialysis described in the section dealing with chromatographic isolation of this protein. The test results were always identical to those obtained after dialysis, indicating that this procedure had no major damaging effect on the Lp(a) factor.

All precipitates between anti-Lp(a) serum and Lp(a+) human serum, or between the antiserum and solutions of isolated Lp(a+)  $\beta$  lipoprotein could be stained by protein and lipid dyes, and the presence of esterase could be demonstrated. The Lp(a) factor is therefore a lipoprotein possessing esterase activity.

Lp(a+) and Lp(a-) human sera were tested in double diffusion experiments against unabsorbed rabbit immune sera. When the immune sera contained anti-Lp(a) antibody, a spur could usually be seen between the precipitates formed by Lp(a+) and Lp(a-) human sera, indicating that the Lp(a+) serum possessed at least one antigenic component more than the Lp(a-) sera (Fig 3).

In some experiments, an additional precipitate near the well containing Lp(a+) serum developed instead of a spur (Fig 4).

The additional line of precipitation showed a reaction of identity with the precipitate obtained with anti-Lp(a) serum. This experiment demonstrated that the Lp(a) factor is a separate antigenic component, different from the rest of the  $\beta$ -lipoprotein. The additional precipitate observed in some experiments, might even indicate that the Lp(a) factor is found in a separate group of  $\beta$  lipoprotein molecules.

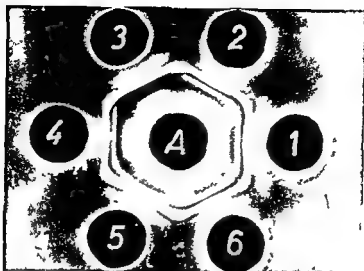


Fig. 5

Precipitation pattern between unabsorbed rabbit immune serum (well 4) and  $Lp(a+)$  (wells 1, 3 and 5) and  $Lp(a-)$  (wells 2, 4 and 6) human sera. Note additional precipitation line adjacent to wells 1, 3 and 5. The serum of well 5 had been stored for 9 months; all other sera were fresh.

#### *Qualitative Constancy of the $Lp(a)$ Factor in Serum of $Lp(a+)$ Individuals*

Sera from the panel donors, from several different bleedings in the course of a 2-year period, were tested for the  $Lp(a)$  factor.

From 21 additional individuals, blood was drawn 3 times at intervals of one week, and the sera submitted to  $Lp$  tests.

From 8 individuals, three blood samples were drawn on one day: the first in the morning when the individuals were fasting, the second 4 hours after breakfast and the third 3 hours after a high fat meal.

In all these experiments, serum from the same individual always gave the same reaction when tested against anti  $Lp(a)$ -serum. It was concluded that the  $Lp(a)$  factor is constantly present in the serum of  $Lp(a+)$  individuals, and that the result of the  $Lp$  test of one individual's serum is not influenced by fasting or high fat intake.

#### *The Effect of Repeated Freezing and Thawing on the $Lp(a)$ Factor*

Fifty fresh  $Lp(a+)$  human sera were subjected to repeated freezing and thawing. The sera were thawed once a day and put back into the deepfreeze at  $-25^{\circ}\text{C}$  as soon as they had all thawed completely. They were tested against anti- $Lp(a)$  serum after different numbers of thawings, and the reactions recorded. After repeated thawings some of the sera gave very weak reactions. Even the precipitates that were close to the limit of visibility were recorded as positive reactions. The results were also recorded in this way when testing the effect of storage under different conditions, described in the following sections. Thus negative



Fig 5

The effect of repeated freezings and thawings on the Lp(a) factor 50 Lp(a+) human sera were tested against anti Lp(a) serum after different numbers of thawings

reaction always means that no precipitate could be seen, whereas sera giving very weak and doubtful precipitates are found among those recorded as reacting positively after different lengths of storage time. The results of the Lp tests after different numbers of thawings are shown in Fig 5.

It will be seen that after the tenth thawing one of the Lp(a+) sera did not react visibly with the antiserum any more, and by later tests, the number of sera still reacting positively decreased steadily. When the sera had been thawed 100 times, only 5 sera still reacted positively.

It was thus evident that the process of freezing and thawing is damaging to the Lp(a) factor.

#### *The Effect of Storage at $-25^{\circ}\text{C}$ on the Lp(a) Factor*

50 Lp(a+) sera from freshly drawn blood samples were stored at  $-25^{\circ}\text{C}$  for 12 months and tested again for the Lp(a) factor. Only 34 sera reacted positively with the antiserum after 12 months of storage. During the storage period, the sera treated



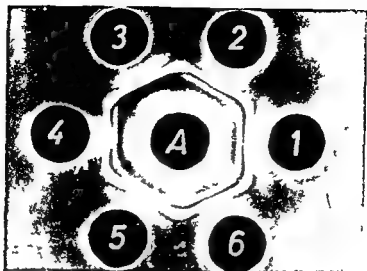


Fig. 4

Precipitation pattern between unabsorbed rabbit immune serum (well 4) and  $I p(a+)$  (wells 1, 3 and 5) and  $I p(a-)$  (wells 2, 4 and 6) human sera. Note additional precipitation line adjacent to wells 1, 3 and 5. The serum of well 5 had been stored for 9 months; all other sera were fresh.

#### *Qualitative Constancy of the $Lp(a)$ Factor in Serum of $I p(a+)$ Individuals*

Sera from the panel donors, from several different bleedings in the course of a 2 year period, were tested for the  $Lp(a)$  factor.

From 21 additional individuals, blood was drawn 3 times at intervals of one week, and the sera submitted to  $Lp$  tests.

From 8 individuals, three blood samples were drawn on one day: the first in the morning when the individuals were fasting, the second 4 hours after breakfast and the third 3 hours after a high fat meal.

In all these experiments, serum from the same individual always gave the same reaction when tested against anti- $Lp(a)$ -serum. It was concluded that the  $Lp(a)$  factor is constantly present in the serum of  $I p(a+)$  individuals, and that the result of the  $I p$  test of one individual's serum is not influenced by fasting or high fat intake.

#### *The Effect of Repeated Freezing and Thawing on the $I p(a)$ Factor*

Fifty fresh  $I p(a+)$  human sera were subjected to repeated freezing and thawing. The sera were thawed once a day, and put back into the deepfreeze at  $-25^{\circ}C$  as soon as they had all thawed completely. They were tested against anti- $Lp(a)$  serum after different numbers of thawings, and the reactions recorded. After repeated thawings some of the sera gave very weak reactions. Even the precipitates that were close to the limit of visibility were recorded as positive reactions. The results were also recorded in this way when testing the effect of storage under different conditions, described in the following sections. Thus negative

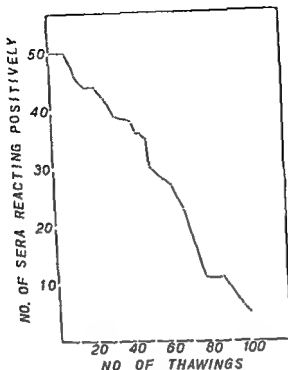


Fig 5

The effect of repeated freezings and thawings on the Lp(a) factor 50 Lp(a+) human sera were tested against anti Lp(a) serum after different numbers of thawings

reaction always means that no precipitate could be seen, whereas sera giving very weak and doubtful precipitates are found among those recorded as reacting positively after different lengths of storage time. The results of the Lp tests after different numbers of thawings are shown in Fig 5.

It will be seen that after the tenth thawing one of the Lp(a+) sera did not react visibly with the antiserum any more, and by later tests, the number of sera still reacting positively decreased steadily. When the sera had been thawed 100 times, only 5 sera still reacted positively.

It was thus evident that the process of freezing and thawing is damaging to the Lp(a) factor.

#### *The Effect of Storage at $-25^{\circ}\text{C}$ on the Lp(a) Factor*

50 Lp(a+) sera from freshly drawn blood samples were stored at  $-25^{\circ}\text{C}$  for 12 months and tested again for the Lp(a) factor. Only 34 (68 per cent) of the sera gave a visible precipitate against the antiserum after this period of storage. These sera had not been thawed during the period between the first and the second testing. 35 Lp(a-) sera treated

in the same way were also tested. The results of the first and second tests were identical.

In addition, sera which had been stored at  $-25^{\circ}\text{C}$  for different lengths of time were tested. These sera had not been submitted to Lp typing in the fresh state. The sera were divided into groups according to the period of storage, and the number of Lp(a+) and Lp(a-) sera in each group was compared by means of "fourfold" tables (Hill 1961, p. 172) with the numbers found in a material of freshly drawn sera from the same population (Marburg, Germany). The results are shown in Table 1, where the  $\chi^2$  for each comparison is included.

TABLE 1

*The Results of Lp Tests of Sera Stored for Different Length of Time at  $-25^{\circ}\text{C}$*

| Storage time months | Number of sera |             |              | $\chi^2$ | (D.F.)            |
|---------------------|----------------|-------------|--------------|----------|-------------------|
|                     | Total          | Lp(a+)      | Lp(a-)       |          |                   |
| 0                   | 301            | 98 (32.56%) | 203 (67.44%) | —        | —                 |
| 1-3                 | 95             | 30 (31.58%) | 65 (68.42%)  | 0.0027   | (0.95 < P < 0.98) |
| 3-9                 | 92             | 20 (21.74%) | 72 (78.26%)  | 3.4276   | (0.05 < P < 0.10) |
| 9-15                | 88             | 12 (13.64%) | 76 (86.36%)  | 11.1057  | (P < 0.001)       |
| 15-21               | 100            | 11 (11.00%) | 89 (89.00%)  | 16.5525  | (P < 0.001)       |

The  $\chi^2$  was calculated as recommended by Hill (1961, p. 172), by comparing each group of stored sera with the fresh sera (storage time 0).

In the groups of sera which had been stored for 9-15 and 15-21 months, the number of Lp(a+) sera was significantly lower than in the material of fresh sera. The most probable explanation is that a number of sera from Lp(a+) individuals had lost their precipitating ability during the period of storage.

130 human sera which had been stored at  $-25^{\circ}\text{C}$  for 6 years were tested. None of these sera reacted positively with anti-Lp(a)-serum, probably because the Lp(a) factor had lost its ability to form a visible precipitate.

12 of these sera were tested against anti- $\beta$ -lipoprotein-serum. All of the sera gave distinct precipitates, indicating that at least a part of the  $\beta$ -lipoprotein still possessed its ability to react with an immune serum.

Samples of the 20 panel sera were tested after storage at  $-25^{\circ}\text{C}$  for periods of different lengths. When the Lp(a+) sera had been stored for about 5-6 months, the precipitates formed with anti-Lp(a)-serum were usually weaker than those obtained with fresh sera. After 8-9 months of storage, some of the samples of Lp(a+) sera had lost their precipitating ability against specific antiserum, but samples still reacting after 2 years of storage were also found.

On two occasions it was observed that samples of Lp(a+) sera had lost their ability to form visible precipitates after 5 months of storage at  $-25^{\circ}\text{C}$ .

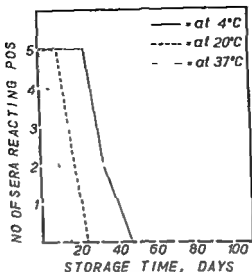


Fig 6

The effect of storage at different temperatures without any added preservative on the Lp(a) factor. Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time

It was concluded that the results of the Lp testing will probably provide correct results only when the sera have been stored at  $-25^{\circ}\text{C}$  for less than about 5 months

No preservative had been added to the sera described in this and the preceding sections

#### *The Effect of Storage at Different other Temperatures and of the Addition of Merthiolate on the Lp(a) Factor*

Samples of 5 Lp(a+) and 5 Lp(a-) human sera, were stored at  $4^{\circ}$ ,  $20^{\circ}$  and at  $37^{\circ}\text{C}$ . Samples of the same sera to which merthiolate in a final concentration of 1/10 000 (w/v) had been added, were stored under identical conditions. The sera were tested for the Lp(a) factor several times during the period of storage, and the results recorded. In Fig 6 the number of Lp(a+) sera still reacting positively at different times is shown for each of the three temperatures

The results found when merthiolate had been added to the sera are shown in Fig 7

Storage at  $37^{\circ}\text{C}$  gave identical results, whether merthiolate was present or not, whereas merthiolate was found to have a preserving effect on the Lp(a) factor at the two lower temperatures

No precipitate was observed between anti-Lp(a)-serum and any of the Lp(a-) sera in these tests

in the same way were also tested. The results of the first and second tests were identical.

In addition, sera which had been stored at  $-25^{\circ}\text{C}$  for different lengths of time were tested. These sera had not been submitted to Lp typing in the fresh state. The sera were divided into groups according to the period of storage, and the number of Lp(a+) and Lp(a-) sera in each group was compared by means of "fourfold" tables (Hill 1961, p. 172) with the numbers found in a material of freshly drawn sera from the same population (Marburg, Germany). The results are shown in Table 1, where the  $\chi^2$  for each comparison is included.

TABLE 1

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| 9-15                | 88             | 12 (13.64%) | 76 (86.36%)  | 11.1057  | (P < 0.001)       |
| 15-21               | 100            | 11 (11.00%) | 89 (89.00%)  | 16.5525  | (P < 0.001)       |

The  $\chi^2$  was calculated as recommended by Hill (1961, p. 172) by comparing each group of stored sera with the fresh sera (storage time 0).

In the groups of sera which had been stored for 9-15 and 15-21 months, the number of Lp(a+) sera was significantly lower than in the material of fresh sera. The most probable explanation is that a number of sera from Lp(a+) individuals had lost their precipitating ability during the period of storage.

130 human sera which had been stored at  $-25^{\circ}\text{C}$  for 6 years were tested. None of these sera reacted positively with anti-Lp(a)-serum, probably because the Lp(a) factor had lost its ability to form a visible precipitate.

12 of these sera were tested against anti- $\beta$  lipoprotein-serum. All of the sera gave distinct precipitates, indicating that at least a part of the  $\beta$ -lipoprotein still possessed its ability to react with an immune serum.

Samples of the 20 panel sera were tested after storage at  $-25^{\circ}\text{C}$  for periods of different lengths. When the Lp(a+) sera had been stored for about 5-6 months, the precipitates formed with anti-Lp(a)-serum were usually weaker than those obtained with fresh sera. After 8-9 months of storage, some of the samples of Lp(a+) sera had lost their precipitating ability against specific antiserum, but samples still reacting after 2 years of storage were also found.

On two occasions it was observed that samples of Lp(a+) sera had lost their ability to form visible precipitates after 5 months of storage at  $-25^{\circ}\text{C}$ .

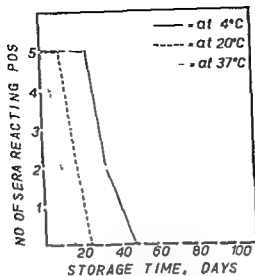


Fig 6

The effect of storage at different temperatures, without any added preservative on the Lp(a) factor. Samples of 5 Lp(a+) sera were tested against anti Lp(a)-serum after different lengths of storage time.

It was concluded that the results of the Lp testing will probably provide correct results only when the sera have been stored at  $-25^{\circ}\text{C}$  for less than about 5 months.

No preservative had been added to the sera described in this and the preceding sections.

#### *The Effect of Storage at Different Other Temperatures and of the Addition of Merthiolate on the Lp(a) Factor*

Samples of 5 Lp(a+) and 5 Lp(a-) human sera, were stored at  $4^{\circ}\text{C}$ ,  $20^{\circ}\text{C}$  and at  $37^{\circ}\text{C}$ . Samples of the same sera to which merthiolate in a final concentration of 1:10 000 (w/v) had been added, were stored under identical conditions. The sera were tested for the Lp(a) factor several times during the period of storage, and the results recorded. In Fig 6 the number of Lp(a+) sera still reacting positively at different times is shown for each of the three temperatures.

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No precipitate was observed between anti-Lp(a)-serum and any of the Lp(a-) sera in these tests.

in the same way were also tested. The results of the first and second tests were identical.

In addition, sera which had been stored at  $-25^{\circ}\text{C}$  for different lengths of time were tested. These sera had not been submitted to Lp typing in the fresh state. The sera were divided into groups according to the period of storage, and the number of Lp(a+) and Lp(a-) sera in each group was compared by means of "fourfold" tables (Hill 1961, p. 172) with the numbers found in a material of freshly drawn sera from the same population (Marburg, Germany). The results are shown in Table 1, where the  $\chi^2$  for each comparison is included.

TABLE 1

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|------------------------|----------------|-------------|--------------|----------|-------------------|
|                        | Total          | Lp(a+)      | Lp(a-)       |          |                   |
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| 1-3                    | 95             | 30 (31.58%) | 65 (68.42%)  | 0.0027   | (0.95 < P < 0.98) |
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| 15-21                  | 100            | 11 (11.00%) | 89 (89.00%)  | 16.5525  | (P < 0.001)       |

The  $\chi^2$  was calculated as recommended by Hill (1961, p. 172), by comparing each group of stored sera with the fresh sera (storage time 0).

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On two occasions it was observed that samples of Lp(a+) sera had lost their ability to form visible precipitates after 5 months of storage at  $-25^{\circ}\text{C}$ .

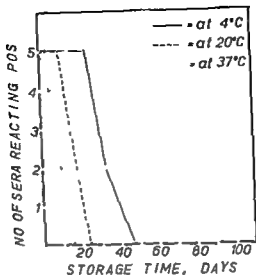


Fig 6

The effect of storage at different temperatures without any added preservative on the Lp(a) factor. Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time

It was concluded that the results of the Lp testing will probably provide correct results only when the sera have been stored at  $-25^{\circ}\text{C}$  for less than about 5 months

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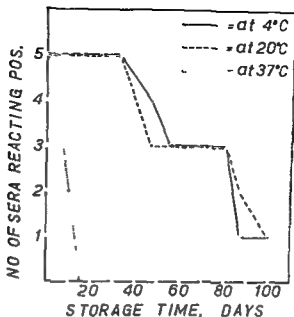


Fig 7

The effect of storage at different temperatures on the Lp(a) factor when merthiolate had been added to the sera as a preservative. Samples of 5 Lp(a+) sera were tested against anti Lp(a) serum after different lengths of storage time.

### *The Effect of Heat on the Lp(a) Factor*

Samples of two Lp(a+) sera and one Lp(a-) serum were heated for 15 minutes at 40°, 50°, 60° and 70° C respectively. After heating, the samples were tested against anti-Lp(a)-serum and anti- $\beta$ -lipoprotein-serum. The samples of the Lp(a+) sera which had been heated to 70° C did not react visibly with anti Lp(a)-serum, whereas those heated to lower temperatures formed distinct precipitates. None of the samples of the Lp(a-) serum formed a precipitate when tested against anti Lp(a)-serum.

All heated serum samples were precipitated by anti- $\beta$  lipoprotein-serum.

### *Studies on the Antigenicity of the Lp(a) Factor*

To investigate whether the Lp(a) factor might have led to the production of isoantibodies in patients who had received multiple blood transfusions, sera of 35 such patients, 5 of type Lp(a+), 30 of type Lp(a-), were tested. All the patients had received more than 5 and some of them more than 40 transfusions.

The tests were performed on agar slides against the 20 panel sera. No anti-Lp(a) antibody was demonstrable in these sera. The finding of isoprecipitins with other specificities in some of these sera will be reported elsewhere.

It was concluded that isoimmunization with the Lp(a) factor, if it

occurs at all, is probably rare. Studies on the heteroantigenic properties of the  $\text{Ip(a)}$  factor are reported in another paper (Berg 1965 a).

## DISCUSSION

The results presented provide evidence that the  $\text{Lp(a)}$  factor is a separate antigenic component within human serum  $\beta$ -lipoprotein. It is also possible that the  $\text{Lp(a)}$  factor is located on a separate group of lipoprotein molecules, different from the rest of the  $\beta$ -lipoprotein molecules. This finding is consistent with earlier reports of immunological heterogeneity of the  $\beta$  lipoproteins (Gullin 1953, Aladjem, Lieberman & Gofman 1957, Aladjem & Campbell 1957), and with the detection of Berg (1964 a) that the  $\text{Lp(r)}$  and  $\text{Ag(a)}$  factors are located on different  $\beta$  lipoprotein molecules.

A true, antigenic difference between  $\text{Lp(a+)}$  and  $\text{Ip(a-)}$  human sera is also indicated by the striking difference in the effect of absorption with  $\text{Lp(a+)}$  and  $\text{Lp(a-)}$  human sera (see Berg 1963, Table 1). Further, no anti  $\text{Lp(a)}$  antibody could be demonstrated in the sera of rabbits immunized with human serum or  $\beta$ -lipoprotein of type  $\text{Ip(a-)}$  (Berg 1965 a). Finally, the qualitative constancy of the  $\text{Lp(a)}$  factor in the serum of  $\text{Lp(a+)}$  individuals supports the assumption of a true antigenic difference. A direct comparison between the amount of  $\beta$  lipoprotein and the  $\text{Ip}$  type has, however, not been performed.

It seems reasonable to presume that the  $\text{Lp(a)}$  factor constitutes a comparatively small part of the total  $\beta$ -lipoprotein.

The instability of the  $\text{Lp(a)}$  factor is consistent with present knowledge of the degradation of serum lipoproteins (Lindgren & Nichols 1960, Briner, Ruddle & Cornwell 1959). The present data may indicate that the  $\text{Lp(a)}$  factor is more easily degraded than the rest of the  $\beta$  lipoproteins.

The protecting effect of the addition of merthiolate to the human sera stored at  $4^\circ$  and  $20^\circ \text{C}$  indicates that bacterial action may play an important part in the degradation of the  $\text{Lp(a)}$  factor. This is consistent with the findings made by Briner, Ruddle & Cornwell (1959) with lipoprotein fractions.

From the data obtained on the stability of the  $\text{Lp(a)}$  factor, it is evident that human sera should be tested for the  $\text{Lp(r)}$  factor in as fresh a state as possible.

## SUMMARY AND CONCLUSIONS

1 The  $\text{Lp(r)}$  factor is a separate, antigenic component perhaps even a separate protein, within the  $\beta$ -lipoproteins of  $\text{Lp(a+)}$  individuals. It probably constitutes a comparatively small part of the total  $\beta$ -lipoprotein.

2 The  $\text{Ip(r)}$  factor belongs to the lipoproteins of density class 1.019-1.063.

3 Studies on the stability of the Lp(a) factor are presented From these experiments, it is evident that the factor is degraded by storage, even in the frozen state, and by freezings and thawings The necessity of using fresh human sera for the Lp typing is stressed

4 Isoimmunization with the Lp(i) factor, in patients receiving multiple blood transfusions, has so far not been observed.

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## STUDIES ON THE REACTION BETWEEN Lp(a+) HUMAN SERA AND ANTI-Lp(a)-SERA FROM RABBITS

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The study of the genetically determined Lp(a) factor of human serum  $\beta$  lipoprotein (Berg 1963), is at present possible only by means of rabbit immune sera. The absorbed immune sera, anti-Lp(a)-sera, react in agar gel double diffusion tests (Ouchterlony 1958) only with human sera possessing the Lp(a) factor (Lp(a+) human sera), and not with those lacking it (Lp(a-) human sera). When equal parts of undiluted Lp(a+) human serum and anti-Lp(a)-serum are allowed to diffuse against each other at 37° C in agar gel of standard composition (Berg 1963), a distinct precipitate develops after 12-14 hours (see Berg 1963, Fig. 4). It is located one third to half the way from the well containing human serum to that filled with antiserum. The presence of protein, lipid and esterase activity in the precipitate can be demonstrated by different staining techniques. In the present paper the precipitate will be referred to as the Lp(a) precipitate or the Lp(a) reaction.

The method to be used as a standard for typing within the Lp system, should be reliable and practical. Several factors known to influence antigen-antibody reactions must be considered to secure optimal conditions for Lp typing.

The purpose of the present work was to study the effect of different factors on the Lp(a) reaction. The results of the experiments performed serve as a guide for the choice of a standard method for Lp testing.

### MATERIALS AND METHODS

#### Human Sera

Human sera from the standard panel and from other healthy donors (Berg 1964 b) were used. The sera were used within a few weeks of storage at -25° C.

#### Hemolysate from Lp(a-) Individual

Whole blood from Lp(a-) individuals was centrifuged at 1000 g for 10 minutes. The supernatant was pipetted off and used for experiments later described.

### *Anti Lp(a) Sera*

Sera from rabbit No 1 absorbed 2/3, from No 3 absorbed 2/5 and from No 52 absorbed 2/3 with Lp(a-) human serum were used (see Berg 1965 a)

### *Petri Dishes*

ANUMBRA dishes, with a diameter of 7 cm were used

### *Glass Slides*

Washed and polished 5 × 5 cm glass slides (Menzel Glaser) were used

### *Agar*

Rheinagar (Behringwerke AG, Marburg Op.-Nr 18, 141, 142 147 148 150) and Bacto Agar (Difco Laboratories Detroit 1, Mich. Control No 45292) were used. The Bacto Agar was washed as described by Hirschfeld (1960) before use.

### *Agar Cutter*

The wells in the agar gel were punched with a precision cutter with a removable cover and an outlet to a suction pump. With this instrument the agar could be removed from the wells during the process of punching. The cutter was made to order (manufacturer Wilhelm Vogel Giessen Germany).

### *Double Diffusion Tests*

The tests were performed either in Petri dishes filled with 24 ml or on 5 × 5 cm glass slides covered with 2.5 ml agar. The standard composition of the gel and the design of the wells for the reagents were as previously described (Berg 1964 b). In the dishes each well was usually filled with 60 µl, on the slides with 15 µl reagent. When nothing else is particularly stated slides and dishes were kept in a moist chamber at 37° C. Readings of precipitates, recording of reactions and photographic registrations were made as described by Berg (1964 b).

The reliability of the standard method for Lp testing was demonstrated by the fact that the panel sera which were tested numerous times in different experiments always gave the same test results. In addition 212 sera were tested twice on 5 × 5 cm slides in Rheinagar of the standard composition. The results of the two experiments were identical.

### *Staining*

The agar slides were stained according to the methods of Uriel (1960 1961) as previously described (Berg 1964 b).

## EXPERIMENTS AND RESULTS

### *The Effect of Type of Agar and Thickness of Gel Layer on the Lp(a) Reaction*

One per cent (w/v) agar gel of the standard composition was prepared in the following ways

- in Petri dishes, each containing 24 ml Bacto Agar giving a layer 6 mm thick
- on 5 × 5 cm glass slides each covered with 2.5 ml Bacto Agar giving a layer 1 mm thick
- on 5 × 5 cm glass slides, each covered with 2.5 ml Rheinagar giving a layer 1 mm thick

100 normal human sera were tested blindly against anti-I p(a)-serum on each of the two types of agar slides and in the dishes. The 100 sera gave the same test results against anti-Lp(a)-serum in the three sets of experiment. It was concluded that all the three modifications provide reliable results of the Lp tests. The slide technique has the advantage of being more economical with the reagents.

#### *The Effect of the Concentration of Agar in Gel on the Lp(a) Reaction*

The experiments described in this and the following sections were all made in duplicate, and the dishes were observed after 2, 3, 4, 5, 6, 10, 12, 14, 24, 36 and 48 hours, and after 3 and 7 days.

Petri dishes were prepared containing 0.5, 1, 2, 3 and 5 per cent (w/v) Bacto Agar (24 ml) respectively, all other constituents of the gel being as described in the standard procedure. In these dishes, anti-Lp(a)-serum in the central well was allowed to diffuse against Lp(a+) and Lp(a-) human sera from the peripheral wells.

In the dishes where the agar concentration was 0.5-1.5 per cent, a precipitate between Lp(a+) human serum and anti Lp(a)-serum was observed 12 hours after the start of the experiment. When the agar concentration was 2 per cent, a very weak precipitate could be seen after 14 hours, and a distinct precipitate after 24 hours. In the dishes containing 3 per cent agar the I p precipitate was observed after 36 hours, and in the dishes with 5 per cent agar, a trace of a precipitate could be seen after 48 hours or more. Once the Lp precipitates had developed, they did not disappear. No precipitates occurred between anti-Lp(a)-serum and Lp(a-) human serum or saline solution.

In the dishes with 0.5 per cent agar, circular precipitates occurred around all wells which had been filled with human sera or immune sera. At this low concentration as well as at the two highest concentrations of agar the gel is difficult to handle. As already mentioned, the precipitates occur later with 2 and 3 per cent agar in the gel than at a concentration of 1.0 or 1.5 per cent.

It was concluded that a gel containing 1.0 or 1.5 per cent agar should be used for the Lp tests. The 1.0 per cent gel has the advantage of being more economical and is therefore preferred.

#### *The Effect of pH in the Agar Gel on the Lp(a) Reaction*

In agar dishes of otherwise standard composition, the pH was varied by addition of buffers with different pH values. The volume of buffer was as given in the standard description. Barbital buffer with an ionic strength of 0.1 was used to obtain a pH of 8.6. For all other pH values, 1.1 M phosphate buffers were used. Dishes with the following pH values were prepared: 5.0, 6.0, 7.0, 8.0 and 8.6. At pH 7.0, 8.0 and 8.6 the I p(a) precipitates could be seen after 12 hours, at pH 5.0 and 6.0 weak precipitates were observed after 14 hours. Circular precipitates

developed around the wells in the dishes containing gel of pH 5.0 and 6.0 and to a lesser degree at pH 8.6. It was therefore concluded that an agar medium with a pH of 7.0-8.0 should be used for the Lp tests.

### *The Effect of Concentration of NaCl in the Agar Gel on the Lp(a) Reaction*

Dishes with the following concentrations of NaCl in the agar gel were prepared. 0, 0.45, 0.9, 2.0, 3.0, 5.0 and 10.0 per cent (w/v). All other constituents of the agar medium were as described in the standard procedure.

The time required for the development of Lp(a) precipitates at the different concentrations of NaCl, is shown in Table 1.

TABLE 1

*Reaction Pattern between Lp(a+) Human serum and Anti Lp(a)-Serum in Agar Gel Double Diffusion Tests at Different Concentrations of NaCl in the Agar Medium*

| Concentration of NaCl in agar gel per cent (w/v) | Reaction time: hours (time after start of experiments) |   |    |    |    |    |     |     |    |     |
|--|--|---|----|----|----|----|-----|-----|----|-----|
|  | 2  | 5 | 10 | 12 | 14 | 24 | 36  | 48  | 72 | 168 |
| 0  | —  | — | —  | ?  | ?  | ?  | ?   | ?   | ?  | ?   |
| 0.45   | —  | — | —  | ?  | ?  | ?  | +   | +   | +  | +   |
| 0.9  | —  | — | —  | +  | +  | +  | +   | +   | +  | +   |
| 2.0  | —  | — | —  | —  | —  | —  | +   | +   | +  | +   |
| 3.0  | —  | — | —  | —  | —  | —  | (+) | +   | +  | +   |
| 5.0  | —  | — | —  | —  | —  | —  | (+) | +   | +  | +   |
| 10.0   | —  | — | —  | —  | —  | —  | —   | (+) | +  | +   |

+ = distinct precipitate

(+) = weak precipitate

— = no visible precipitate

? = reaction impossible to read because of circular precipitates around the wells

When the NaCl concentration was 2.0 per cent or higher the time necessary for the development of Lp(a) precipitates was considerably increased. The precipitates were also distinct at these higher NaCl concentrations.

In the dishes containing agar with no or 0.45 per cent NaCl, large, dense, circular precipitates developed around the wells. These concentric precipitates made it impossible to read the Lp reactions in agar containing no NaCl. In the gel with 0.45 per cent NaCl, the Lp reactions could only be clearly read after 36 hours.

It was concluded that a NaCl concentration of 0.9 per cent (w/v) in the agar gel should be used for the Lp tests.

### *The Effect of Temperature on the Lp(a) Reaction*

All experiments so far described were performed at 37° C. To test the effect of temperature on the Lp(a) reaction, Petri dishes containing

gel of the standard composition were kept at 4, 20 and 37° C. In addition to the readings already mentioned, these dishes were also observed after 16, 18, 20 and 22 hours. Distinct precipitates between Lp(a+) human sera and anti-Lp(a)-sera were observed after 12 hours at 37° C, after 14 hours at 20° C, and after 22 hours at 4° C. No other differences were observed. The precipitates did not disappear once they had developed.

The Lp tests can obviously be performed at any of the three temperatures.

The results of the Lp tests are obtained earlier at 37° C than at lower temperatures, and incubation at 37° C is therefore preferred.

#### *The Effect of Different Ratios of Reagents on the Lp(a) Reaction*

Different amounts of undiluted human serum and anti-Lp(a)-serum were tested on 5 × 5 cm agar slides. When more than 15  $\mu$ l of a reagent was used, the well was refilled as soon as the previously introduced serum had diffused into the surrounding agar gel.

Fifteen  $\mu$ l Lp(a+) human serum in the central well was tested against the following volumes of anti-Lp(a)-serum in the peripheral wells: 5, 10, 15, 20, 40 and 60  $\mu$ l. As a control, the same experiment was also performed with Lp(a-) human serum.

In another experiment, 15  $\mu$ l anti-Lp(a)-serum in the central well was tested against the following amounts of Lp(a+) human serum in the peripheral wells: 5, 10, 15, 20, 40 and 60  $\mu$ l. This experiment was also performed with Lp(a-) human serum.

In both experiments the most favourable reactions with respect to localization, density and time of appearance of the Lp(a) precipitate were found where about equal amounts of antiserum and Lp(a+) human serum reacted with each other. When excess of antiserum was used, the Lp(a) precipitate was located close to the well containing human serum and was very difficult to read. When, however, 60  $\mu$ l Lp(a+) human serum was tested against 15  $\mu$ l anti-Lp(a) serum, the precipitate was still located comparatively far away from the well containing antiserum, and was easy to read. When the smallest amount (5  $\mu$ l) of either Lp(a+) human serum or anti-Lp(a) serum was used, the Lp(a) precipitate was very weak, and required a long time of reaction to develop.

No precipitate between Lp(a-) human serum and anti-Lp(a)-serum was observed in these experiments.

It was concluded that equal parts of human sera, and the anti-Lp(a)-serum employed in the described experiments, should be used for testing for the Lp(a) factor.

Experiments similar to those dealt with in this section should be performed with each new anti-Lp(a)-serum to ascertain which ratio between antiserum and human serum should be used in the testing for



developed around the wells in the dishes containing gel of pH 5.0 and 6.0 and to a lesser degree at pH 8.0. It was therefore concluded that an agar medium with a pH of 7.0-8.0 should be used for the Lp tests.

### *The Effect of Concentration of NaCl in the Agar Gel on the Lp(a) Reaction*

Dishes with the following concentrations of NaCl in the agar gel were prepared: 0, 0.45, 0.9, 2.0, 3.0, 5.0 and 10.0 per cent (w/v). All other constituents of the agar medium were as described in the standard procedure.

The time required for the development of Lp(a) precipitates at the different concentrations of NaCl, is shown in Table 1.

TABLE 1

*Reaction Pattern between Lp(a+) Human serum and Anti Lp(a) Serum in Agar Gel Double Diffusion Tests at Different Concentrations of NaCl in the Agar Medium*

| (concentration of NaCl in agar gel per cent (w/v)) | Reaction time: hours (time after start of experiments) |   |    |    |    |    |    |     |     |     |
|--|--|---|----|----|----|----|----|-----|-----|-----|
|  | 2  | 5 | 10 | 12 | 14 | 24 | 36 | 48  | 72  | 168 |
| 0  | —  | — | —  | ?  | ?  | ?  | ?  | ?   | ?   | ?   |
| 0.45   | —  | — | —  | ?  | ?  | ?  | +  | +   | +   | +   |
| 0.9  | —  | — | —  | +  | +  | +  | +  | +   | +   | +   |
| 2.0  | —  | — | —  | —  | —  | —  | +  | +   | +   | +   |
| 3.0  | —  | — | —  | —  | —  | —  | —  | (+) | +   | +   |
| 5.0  | —  | — | —  | —  | —  | —  | —  | (+) | +   | +   |
| 10.0   | —  | — | —  | —  | —  | —  | —  | —   | (+) | +   |

+ = distinct precipitate

(+) = weak precipitate

— = no visible precipitate

? = reaction impossible to read because of circular precipitates around the wells

When the NaCl concentration was 2.0 per cent or higher the time necessary for the development of Lp(a) precipitates was considerably increased. The precipitates were also distinct at these higher NaCl concentrations.

In the dishes containing agar with no or 0.45 per cent NaCl large, dense, circular precipitates developed around the wells. These concentric precipitates made it impossible to read the Lp reactions in agar containing no NaCl. In the gel with 0.45 per cent NaCl, the Lp reactions could only be clearly read after 36 hours.

It was concluded that a NaCl concentration of 0.9 per cent (w/v) in the agar gel should be used for the Lp tests.

### *The Effect of Temperature on the Lp(a) Reaction*

All experiments so far described were performed at 37° C. To test the effect of temperature on the Lp(a) reaction, Petri dishes containing

### *The Effect of Complement on the Ip(a) Reaction*

Fresh panel sera were tested against an anti Ip(a) serum. Samples of the panel sera and the anti Ip(a) serum were kept at 56° C for 30 minutes in order to inactivate complement. Samples of panel sera treated in this way were thereafter tested against untreated as well as inactivated anti Ip(a) serum. In addition untreated samples of panel sera were tested against inactivated anti Ip(a) serum. In all these experiments the test results were identical to those obtained in the first test when only untreated reagents were used. It was concluded that the presence of active complement is not necessary for the development of the Ip(a) precipitate.

### *A Special Technique for the Testing of Sera from Hemolysed Blood Samples*

When sera from hemolysed blood samples are tested dense circular precipitates develop around the wells. These precipitates make the reading of the Ip(a) reaction difficult or impossible. A procedure separating the  $\beta$  lipoprotein from the disturbing components of hemolysed blood cells prior to diffusion against anti Ip(a) serum was therefore developed. The amounts of sera were the same as those of the standard technique on glass slides. The following method combining electrophoretic separation of  $\beta$  lipoprotein from the constituents of the hemolyte at pH 7.0 with diffusion against anti Ip(a) serum was applied.

5 x 5 cm slides were covered with 2 ml 1 per cent (w/v) Bacto Agar in a 0.05 M sodium phosphate buffer of pH 7.0. On these slides 3 wells were cut in the gel, 2 near one end of the slide, the third more centrally as shown in Fig. 1.

Each well had a diameter of 4 mm, the distance from the circumference of the central well to the other wells was 10 mm, and the distance between the circumferences of the latter two wells 8 mm.

The two wells near the end of the slide were each filled with 15  $\mu$ l human serum to be tested, and the electrophoresis was started. The electrode vessels contained 0.05 M sodium phosphate buffer with pH 7.0 and the filter paper (Whatman No. 3) connecting electrode buffer compartments with the agar slides were soaked in this buffer before use. The negative pole was at the end near the human sera. The electrophoresis was carried on for 2½ hours at 100 V and 35 mA. During the electrophoresis  $\beta$  lipoprotein moved towards the positive pole and was thereby separated from the main components of the hemolyte which moved towards the negative pole. After electrophoresis 15  $\mu$ l anti Ip(a) serum was introduced into the central well, the slides were kept in a moist chamber at 37° C, and the reactions read after 1, 2 and 3 days (no precipitates took more than 2 days to develop). The human sera were classified as Ip(a+) or Ip(a-) depending upon whether a line

the Lp(a) factor. So far, all the anti-Lp(a)-sera used for routine testing have given optimal reactions when equal parts of antiserum and human serum were used. These results were also expected, as the absorption ratios for the typing reagents were selected from experiments with equal parts of human serum and absorbed immune serum.

#### *The Effect of Dilution of the Reagents on the Lp(a) Reaction*

Anti-Lp(a)-serum was diluted with a 0.85 per cent saline solution. The following dilutions were used: 1/1 (undiluted), 1/2, 1/4, 1/8, 1/16, 1/32, 1/64. Five Lp(a+) human sera and one Lp(a-) serum were diluted in the same way. Corresponding dilutions of antiserum and human sera were tested against each other in double diffusion experiments on glass slides. The agar slides were observed after 1, 2, 3 and 7 days, and thereafter weekly for 4 weeks. When undiluted (1/1 dilution) reagents were tested against each other, distinct precipitates were observed against all Lp(a+) human sera after 1 day. On the slides where reagents diluted 1/2 were tested, the Lp(a) reactions required 2 days to develop. After 3 days, a precipitate had been formed by 4 of the 5 Lp(a+) human sera in the experiment where the reagents were diluted 1/4, and after 7 days the last Lp(a+) human serum also reacted positively. No precipitate was observed when the reagents were diluted 1/8 or more. The precipitates formed by Lp(a+) human sera did not disappear once they had developed. No precipitate was observed between antiserum and the Lp(a-) human serum in any of the experiments.

It was concluded that undiluted reagents should be used for the Lp tests.

#### *The Effect of Merthiolate on the Lp(a) Reaction*

Agar dishes of the standard composition apart from not containing merthiolate were prepared, and Lp(a+) and Lp(a-) human sera were tested against anti-Lp(a)-serum. The test results were identical to those obtained in gel containing merthiolate. It was thus obvious, that the merthiolate added to the gel does not interfere with the Lp(a) reaction.

Merthiolate was added to a sample of anti-Lp(a)-serum and to samples of each of the 20 panel sera to a final concentration of 1/10 000 (w/v). In standard agar dishes, anti-Lp(a)-serum containing merthiolate was tested against the panel sera with and without merthiolate, and anti-Lp(a)-serum without merthiolate was tested against panel sera containing merthiolate. In these experiments the test results were always identical to those obtained with the panel sera and anti-Lp(a)-serum, when none of the reagents contained merthiolate. It was concluded that merthiolate added to the reagents in a concentration of 1/10 000 (w/v) had no damaging effect on the Lp(a) reaction.

or in whole human serum. This precipitate therefore does not represent any source of error in the experiments employing whole human serum. With the standard composition of the gel, and at reaction temperature of 37° C, the mentioned non-immune precipitate near a well containing isolated  $\beta$  lipoprotein occurs about 3 hours after the start of the experiment, and disappears 7-9 hours later. It has therefore usually disappeared when the Lp(a) precipitate develops. These reaction conditions are therefore also useful for the study of the Lp type of isolated  $\beta$  lipoprotein. When the concentration of NaCl in the gel is 2 per cent or higher, the development of the described non-immune precipitate is prevented.

Optimal conditions for the Lp(a) reaction were found when equal amounts of undiluted antiserum and human serum were used, but the precipitate also developed after some dilution of the reagents, and when different proportions of the reacting sera were used. Some dilution of the sera by admixture of saline from the pipettes should therefore not affect the results of the tests. It is further reason to believe that variations in the amount of  $\beta$ -lipoprotein between individual human sera do not influence the test results. Experiments with different amounts of sera and with diluted sera should, however, be performed with all new antisera to ascertain that the results are similar to those described in the present paper.

The test technique gives reliable results when fresh human sera are used. During storage, the Lp(a) factor deteriorates, even at -25° C (Berg 1964 b). The Lp(a) factor is better preserved when merthiolate is added. This phenomenon, as well as the presence of the factor in the serum of different bleedings of the same individual, are dealt with in another paper (Berg 1964 b).

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The loss of reactivity of human sera on storage could indicate that the presence of active complement was essential for the Lp(a) reaction. The experiments prove, however, that active complement is not essential for the reaction. The change in reactivity of sera during storage is probably due to lability of the  $\beta$  lipoprotein molecule.

Some of the experiments in the present study sustain the theory that the reaction between anti-Lp(a)-serum and Lp(a+) human serum is

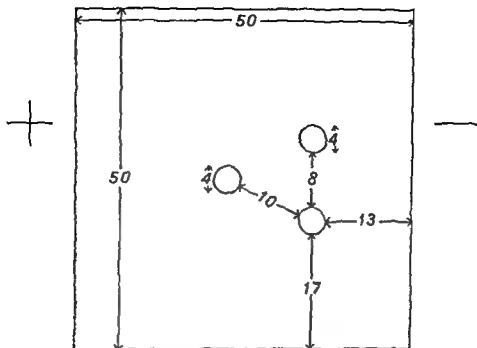


Fig 1

Arrangement of the wells on the agar slide used for the special test method designed for sera from hemolyzed blood samples (see text). The diameter of each well is 4 mm. All measures are in millimetres.

of precipitation could be observed or not (see Berg & Mohr 1963, Fig 2). To test the reliability of this method, 60 human sera already tested with the standard technique, were tested blindly by means of the method described in this section. For each of these sera, 1 drop of hemolysate from a Lp(a—) individual was added to 9 drops of serum, before testing with the present technique.

The test results obtained with the two methods were identical. It was therefore concluded that the procedure described can be used for the Lp testing of sera contaminated by hemolysate.

#### DISCUSSION

The present experiments have shown that the Lp(a) reaction manifests itself over a wide variety of reaction conditions. It can be read at several values of pH and at several concentrations of NaCl and agar in the gel. The use of  $5 \times 5$  cm glass slides instead of dishes has the advantage of being more economical. The composition of the gel described in the standard procedure, and a reaction temperature of  $37^\circ\text{C}$ , are preferred. Under these conditions it is easy to distinguish the Lp(a) reaction from a non-immune precipitate between isolated human serum  $\beta$ -lipoprotein and albumin observed by Berg (1964a). This precipitate was only observed when  $\beta$ -lipoprotein in the isolated state was used, whereas albumin could be present either in the isolated state

or in whole human serum. This precipitate therefore does not represent any source of error in the experiments employing whole human serum. With the standard composition of the gel, and at reaction temperature of 37° C, the mentioned non-immune precipitate near a well containing isolated  $\beta$  lipoprotein occurs about 3 hours after the start of the experiment, and disappears 7-9 hours later. It has therefore usually disappeared when the Lp(a) precipitate develops. These reaction conditions are therefore also useful for the study of the Lp type of isolated  $\beta$  lipoprotein. When the concentration of NaCl in the gel is 2 per cent or higher, the development of the described non immune precipitate is prevented.

Optimal conditions for the Lp(a) reaction were found when equal amounts of undiluted antiserum and human serum were used, but the precipitate also developed after some dilution of the reagents, and when different proportions of the reacting sera were used. Some dilution of the sera by admixture of saline from the pipettes should therefore not affect the results of the tests. It is further reason to believe that variations in the amount of  $\beta$ -lipoprotein between individual human sera do not influence the test results. Experiments with different amounts of sera and with diluted sera should, however, be performed with all new antisera to ascertain that the results are similar to those described in the present paper.

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## TRANSACTIONS OF THE PATHOLOGICAL SOCIETY OF NORWAY

*Meeting February 13, 1964*

*Oslo, Norway*

*Schalt Haudt S D* CURRENT ASPECTS ON THE BIOCHEMISTRY OF COLLAGEN

*Chetremont H Freire* MITOCHONDRIA

A picture shown and commented by *O H Iversen*

*Meeting April 16, 1964*

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*Scarff R W* AN INFORMATION ON ICSP

*Fagerhol M A* THE DETERMINATION OF BLOOD GROUPS IN BLOOD  
DONORS AT NORWEGIAN HOSPITALS

The frequency of errors in blood group determinations in the years 1961-63 have been determined for 52 Norwegian hospitals. In 37 hospitals using standard techniques the ABO grouping failed in 34 of 7321 groupings i.e. 0.46 per cent. The Rh typing failed in 93 of 6009 typings i.e. 1.51 per cent. In 15 hospitals using Eldon cards ABO grouping failed in 6 of 879 groupings i.e. 0.72 per cent. The Rh typing failed in 37 of 874 typings i.e. 4.33 per cent. It is recommended that the ABO and Rh grouping of all new blood donors should be controlled in a blood group laboratory and that the hospitals should perform ABO and Rh typing prior to this control to detect and eliminate systematic errors in the local blood grouping procedure. Only well established techniques which permit adequate controls should be used.

*Loe K* THE PERCENTAGE INCIDENCE OF SEROPOSITIVE SYPHILIS IN  
PREGNANT WOMEN IN NORWAY 1958-1963

The incidence of infectious syphilis has increased in many parts of the world during the last five years in Norway only a small rise during the last year (1963). The frequency of seropositive syphilis in the population may also give some information regarding this problem. In Norway all women are controlled with serological syphilis tests in the pregnancy. In the Sero diagnostic department State Institute of Public Health Oslo blood samples from 173-176 pregnant women were examined by serological syphilis tests during the five year period from May 1,

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1958 to May 1 1963 Sera giving positive standard tests were also tested with the TPI test to exclude false positive reactors

A total of 654 pregnant women had positive standard tests of syphilis of which 183 were TPI negative The average incidence of seropositive syphilis thus being 0.27 per cent (471 pregnant women) during the whole five year period

The annual rate has decreased during the same period from 0.38 per cent in the first year (1/5 58 1/5 59) to 0.17 per cent in the last year The rate for the last year is the lowest rate ever observed in this laboratory The frequency of seropositive syphilis in the five year period is reduced in all age groups also in the youngest groups

The investigation indicates that in this five year period 1958-1963 syphilis has been reduced among the female population in Norway

#### *Myhre E* MALIGNANT MELANOMAS IN CHILDREN

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